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Investigating the Mechanism of Geminin Inactivation

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Thesis submitted for the degree of Doctor of Philosophy

Investigating the Mechanism of Geminin Inactivation

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Abbreviations

2D	2 Dimension
6-DMAP	6-Dimethylaminopurine
ABS-14	Amidosulfobetaine-14
ACN	Acetonitrile
ACS	ARS Consensus Sequences
APC/C	Anaphase Promoting Complex/Cyclosome
ARS	Autonomously Replicating Sequences
ATP	Adenosine Triphosphate
bp	Basepairs
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
Cdc6	Cell Division Cycle 6
CDKs	Cyclin-Dependent Kinases
Cdt1	Cdc10-Dependent Transcript 1
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-Propanesulfonate
CHX	Cyclohexamide
Cryo-EM	Cryogenic Electron Microscopy
D-box	Destruction box
DDK	Dbf4/Drf1 Dependent Kinase
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
<i>E. Coli</i>	<i>Escherichia Coli</i>
EB	Extraction Buffer
EDB-S	Extract Dilution Buffer with Sucrose

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene Glycol Tetraacetic Acid
ER	Energy Regenerator
G1	Gap phase 1
G2	Gap Phase 2
Hbo1	Human acetylase Binding to Orc1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His-Ub	His6-Ubiquitin
IPTG	Isopropyl β -D-1-thiogalactopyranoside
K11O Ub	K11-only Ubiquitin
K11R Ub	Ubiquitin K11R
K48O Ub	K48-only Ubiquitin
K48R Ub	Ubiquitin K48R
LB	Lysogeny broth
LFB1	Licensing Factor Buffer 1
LFB2	Licensing Factor Buffer 2
MBP	Maltose Binding Protein
MBT	Mid Blastula Transition
MCM	Maintenance of Minichromosome
MMR	Modified Marc's Ringer
M-Ub	Methylated Ubiquitin
NIB	Nuclear Isolation Buffer
NIBS	Nuclear Isolation Buffer with Sucrose
NLS	Nuclear localisation signal
NPE	Nucleoplasmic Extract
OC6C1M	ORC-Cdc6-Cdt1-MCM2-7 complex

ORC	Origin Recognition Complex
ORCA	ORC Associated
ori	Replication origin
PAGE	Polyacrylamide Gel Electrophoresis
PIE	Pre-Incubated Extract
PIP	PCNA Interacting Protein box
PBS/T	Phosphate Buffered Saline with Tween 20
PMSF	Phenylmethanesulfonyl Fluoride
Pol ϵ	Polymerase Epsilon
Pre-IC	Pre-Initiation Complex
Pre-LC	Pre-Loading Complex
Pre-RC	Pre-Replicative Complex
PVDF	Polyvinylidene Fluoride
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SDS	Sodium Dodecyl Sulfate
SPF	S-Phase Promoting Factor
SV40	Simian Transforming Virus 40
TFA	Trifluoroacetic acid
UbVS	Ubiquitin Vinyl Sulfone
v:v	Volume per Volume
w:v	Weight per Volume

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Declaration

I, Kevin Creavin, declare that I am the sole author of this thesis, that the research presented here is my own unless there is a clear statement otherwise. None of this research has previously been accepted for a higher degree.

Kevin Creavin

J. Julian Blow

Abstract

DNA replication is regulated throughout the cell cycle to ensure that the entire genome is replicated once per cell cycle. Replication licensing is a key process required for the initiation of DNA replication. Replication licensing refers to the chromatin loading of an essential replication factor, the MCM2-7 helicase. This is loaded onto DNA early in the cell cycle. MCM2-7 stay bound on chromatin until cells enter S-phase where CDKs act upon the MCM2-7 complex leading to the initiation of DNA replication. The processes of replication licensing and initiation are separated during the cell cycle to ensure these activities do not overlap as this can result in the rereplication of DNA. Separation of the two processes enables licensing inhibition prior to the activation of CDKs. A key regulator of MCM loading is a coiled-coil protein called geminin. Geminin potently inhibits licensing by binding and inhibiting the replication licensing factor Cdt1. In somatic cell cycles geminin is degraded by the 26S proteasome to allow licensing to take place. However, in embryonic cell cycles geminin remains stable throughout the cell cycle. This is essential as geminin is a dual function protein. It is required in embryonic cell cycles to maintain pluripotency; therefore it must remain stable in these cell types. It is also a potent inhibitor of DNA replication; therefore it must be subjected to some form of inactivation.

The aim of this project was to identify the molecular mechanism of geminin inactivation in *Xenopus* egg extracts. Experiments undertaken during this project have demonstrated that geminin is both stable and inactive in *Xenopus* egg extracts. In addition some factors essential for the inactivation of geminin have been identified. The kinetics of inactivation have been determined and it has been demonstrated that interphase inactive geminin is biochemically distinct from active metaphase geminin.

1.0 Introduction

1.1 The Cell Cycle

When a eukaryotic cell commits to proliferation it is taking on the monumental task of precisely duplicating billions of basepairs (bp) of deoxyribonucleic acid (DNA) and correctly segregating the entire genome into two daughter cells. These processes must be completed flawlessly with almost no room for error; this is particularly so in multicellular organisms in which inherited errors in the genome can have profoundly catastrophic effects resulting in the development of cancer. However, cells faithfully carry out these processes and billions undergo cell division every day in the human body. This highlights the fact that cells contain robust mechanisms to ensure the essential tasks of DNA replication and segregation are carried out precisely and accurately with close to zero error.

The cell division cycle is essentially an ordered sequence of events which progresses in only one direction to ensure the timely and precise duplication of DNA, followed by segregation of the replicated DNA into two daughter cells (Figure 1). When a cell enters the cell cycle it is fully committed to achieving these goals as there is no way to reverse the process and the only way to interrupt the cycle is to enter apoptosis, controlled cell death. The cell cycle is highly regulated using intricate feed-forward and feed-back mechanisms to ensure essential processes are carried out sequentially and in the correct temporal order. To prevent inappropriate progression, the cell cycle contains robust checkpoints where key criteria must be satisfied for progression, and cells which fail to satisfy these criteria ultimately enter apoptosis.

DNA is replicated in a period of interphase termed S-phase, which is preceded and followed by gap phases, G1 and G2 respectively. During G1 of the cycle cells pass a restriction point to ensure the cell is prepared for DNA replication in the following S-

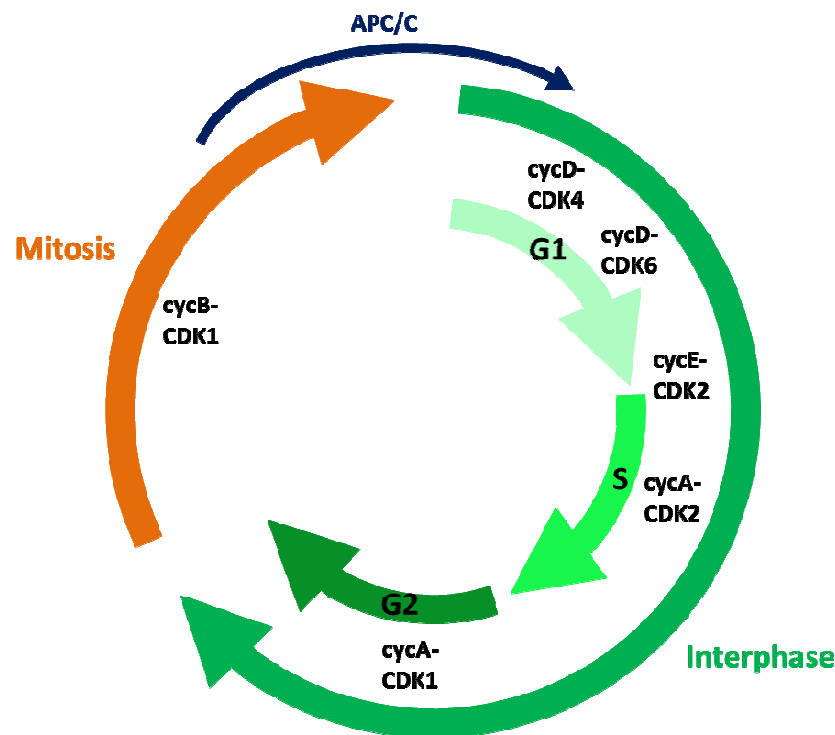


Figure 1: Regulation of the Cell Cycle. Different cyclin CDK pairs drive progression through the cell cycle. During mitosis CDK activity leads to the activation of the APC/C. The APC/C triggers the completion of mitosis and separation of duplicated chromatids into two daughter cells.

phase and during G2 mechanisms or checkpoints ensure the cell and the newly duplicated DNA are prepared for mitosis. During mitosis the duplicated DNA is segregated into two genetically identical daughter cells.

The eukaryotic cell cycle is driven by cyclin-dependent kinases (CDKs). CDKs regulate the activity of many target proteins by phosphorylation, thereby orchestrating the specific activities and processes that occur at different cell cycle stages. CDKs require a coactivator protein, termed cyclin, to form an active CDK-cyclin heterodimer. The number of unique CDKs and cyclins can vary in organisms. Multiple CDK-cyclin pairs are formed to regulate CDK activity, direct substrate specificity and control CDK localisation. The temporal order of the cell cycle is controlled by the sequential expression and degradation of the cyclin proteins. Cyclin levels oscillate during the cell cycle to ensure the correct CDK-cyclin pairs are active in the correct sequence,

activating and inhibiting processes where required, to drive ordered progression through the cell cycle.

The cyclic nature of cyclin expression highlights the importance of timely protein destruction. Ubiquitination, and the subsequent destruction, of target proteins plays a crucial role in the regulation of the cell cycle. During mitosis, the activity of the Anaphase Promoting Complex/Cyclosome (APC/C) E3 ubiquitin ligase causes ubiquitination and degradation of cyclins, resulting in a period of low CDK activity, essentially resetting the cell cycle. CDKs and the APC/C are together considered the masters of the cell cycle (Pines, 2011).

DNA replication initiation is strictly regulated throughout the entire cell cycle. Each basepair of DNA in the genome is faithfully duplicated once and only once per cell cycle. To achieve this, the process of replication initiation is split by the cell cycle into two distinct non-overlapping stages, origin licensing and origin firing. The regulation of origin licensing is the focus of this thesis.

1.2 The Replicon Model

Ever since it became apparent that DNA is the carrier of genetic information one of the fundamental goals of modern biology has been to understand how cells carry out the monumental task of DNA replication. Jacob and Brenner put forward a model 52 years ago, using the properties of bacterial transcription, to describe the molecular mechanism of DNA replication initiation in bacteria. It was proposed that there are two genetic loci essential for replication initiation; a functional protein encoded by an 'initiator' gene would bind a specific 'replicator' sequence of DNA to initiate replication. DNA replication initiated from the replicator would then proceed until the entire genetic element was copied (Jacob and Brenner, 1963). This provided a paradigm for early research on DNA replication and this model was found to hold true for DNA replication

in bacteria and for viruses such as the polyomavirus, Simian Transforming Virus 40 (SV40).

Higher eukaryotic organisms maintain very large genomes with billions of basepairs of DNA packaged as multiple linear chromosomes in the nucleus. In this context the initiation of DNA replication requires a more complex regulation than what was first hypothesised for bacteria. In spite of this, the generalisations of the Replicon Model can be applied to most model organisms and it has been found that significant functional homology is observed in proteins that are involved in the replication of DNA. This early work provided a greater understanding of the molecular mechanisms of DNA replication and the types of proteins required to carry out specific roles in the process.

1.2.1 DNA Replication Initiation in Bacteria

Replication initiation of the *Escherichia coli* (*E. coli*) circular chromosome was found to take place at one site, named the replication origin (ori) (Louarn et al., 1974) and proceeds in a bidirectional process until the entire circular chromosome is replicated. The replication origin was shown to be a single defined DNA sequence termed oriC (Oka et al., 1980). DnaA uses ATP to bind oriC leading to the unwinding of the origin (Sekimizu et al., 1987, Sekimizu et al., 1988). This permits the binding of DnaB-DnaC complex. DnaC deposits DnaB onto the single stranded DNA, which triggers DnaB activation (Wahle et al., 1989a, Wahle et al., 1989b). DnaB forms a hexameric complex with helicase activity and recruits additional replication proteins, such as single stranded binding protein and DNA gyrase (Bramhill and Kornberg, 1988, West, 1996). In terms of the replicon model oriC represents the ‘replicator’, or the site of replication initiation, and DnaA, DnaB and DnaC together represent the ‘initiator’. Work in *E. coli* identified three key roles that could be expected of eukaryotic ‘initiator’

proteins. They must bind to sites of replication initiation, unwind the DNA and serve to recruit additional factors allowing replication (Bramhill and Kornberg, 1988).

1.2.2 DNA Replication Initiation of the SV40 Virus

The SV40 genome consists of a circularised DNA template which contains a single sequence defined replication origin, termed *ori*, which represents the Replicon 'replicator'. SV40 requires a single viral protein, T-antigen, for replication of the virus DNA and hijacks additional replication factors from the host cell. T-antigen carries out multiple functions, first acting as the replicon 'initiator' by binding to the origin of replication and later functioning as a hexameric replicative helicase. The requirement for host cell replication proteins led to the development of an *in vitro* SV40 replication system (Challberg and Kelly, 1979a, Challberg and Kelly, 1979b), which has been invaluable for identifying key replication factors and for early investigations into the molecular mechanisms of eukaryotic DNA replication.

1.3 Eukaryotic Origins of Replication

1.3.1 Requirement for multiple origins

The Replicon model is too simplistic to adequately explain the complexities of DNA replication in higher eukaryotes. One significant difference is that higher organisms have much larger genomes, that are divided amongst multiple linear chromosomes which must be replicated in a relatively short time during S-phase, before the onset of mitosis (Figure 2). For this, a single replication origin is insufficient. It was shown in cultured cells of Chinese hamster ovary and HeLa cells that subsections of certain chromosomes replicated DNA replication at different times during S-phase, suggesting that there are multiple replication start sites or 'origins of replication' within the eukaryotic chromosome (Taylor, 1960, Cairns, 1966, Huberman and Riggs, 1968). In

fact, hundreds or thousands of origins of replication fire within a single eukaryotic S-phase, depending on the cell type and developmental stage.

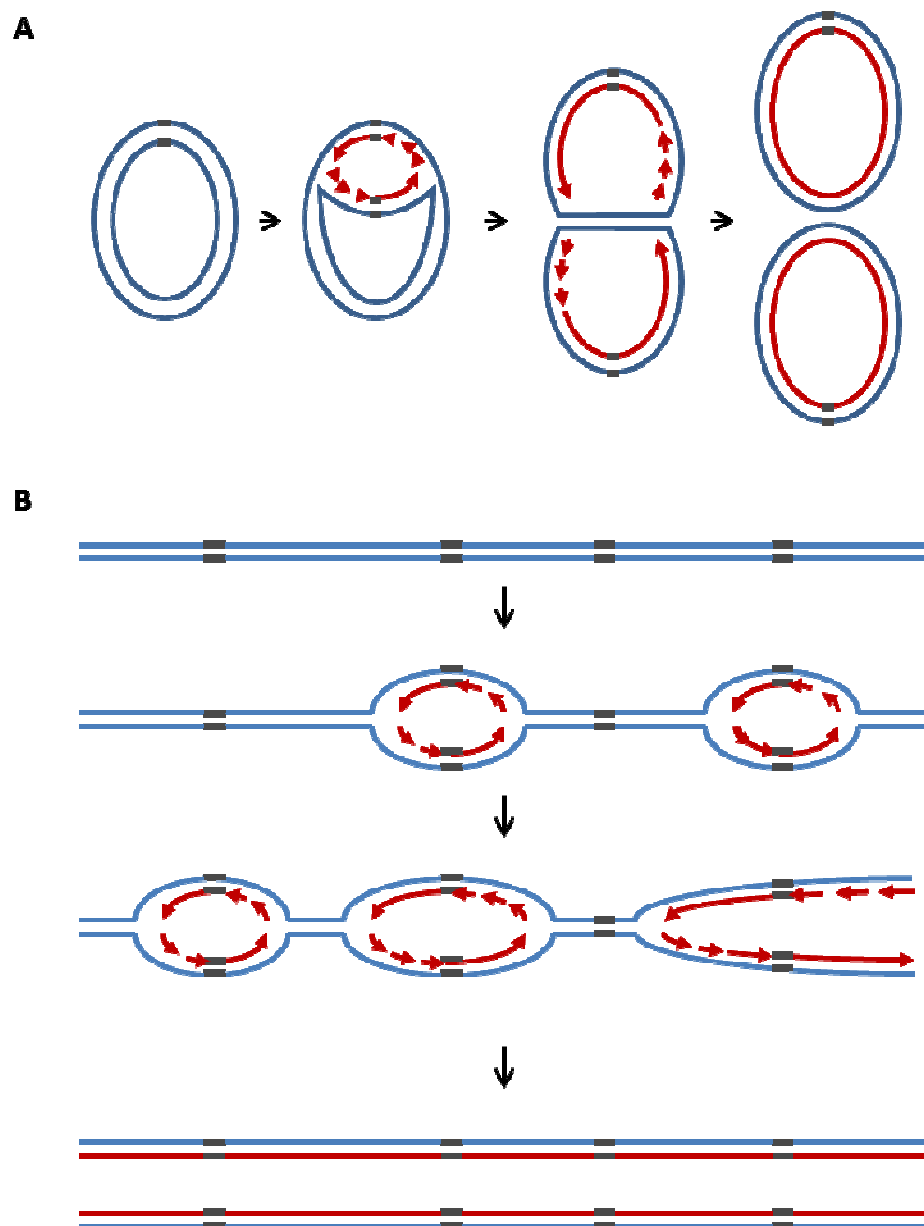


Figure 2: DNA Replication from a Single or Multiple Origins.(A) Replication of a circular chromatid from a single origin as occurs in bacteria. **(B)** Replication of linear a chromosome from multiple origins, as occurs in higher eukaryotes. Origins can fire at different times and some remain dormant and do not fire in the absence of replicative stress.

In addition to the many origins that are used during a single S-phase there is an excess of potential origins which are not used under normal circumstances. The numbers of excess origins have been quantified and depending on the model system there are anywhere from 3-10 fold more origins of replication capable of initiating DNA

replication which are not used (Burkhart et al., 1995, Donovan et al., 1997, Mahbubani et al., 1997, Wong et al., 2011) and are passively replicated during S-phase (Santocanale and Diffley, 1996, Woodward et al., 2006). Subsequent work has shown that these excess origins function as ‘dormant origins’ (Woodward et al., 2006). These origins have theoretically the same likelihood as any other origin to fire in S-phase. Dormant origins are present to provide additional initiation sites in the event of replicative stress-dependent fork stalling during S-phase (Ge et al., 2007). How cells determine whether an origin will fire or not is not well understood, however origin firing appears to be a stochastic process.

1.3.2 Origins of Replication in Yeast

The origins of replication of the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) are the best characterised of all eukaryotic organisms. It has been shown by numerous methods that budding yeast contains approximately 482 origins of replication (Siow et al., 2012, Newman et al., 2013) which contain a consensus DNA sequence. These sequences were first identified as they permitted the replication of transforming plasmids introduced into yeast cells, allowing them to replicate autonomously without incorporation into the chromosome. These sequences are referred to as Autonomously Replicating Sequences (ARSs) (Struhl et al., 1979, Stinchcomb et al., 1979). It was subsequently shown that replication initiates proximal to the ARS element providing further evidence for their role as the origin of replication (Brewer and Fangman, 1987, Huberman et al., 1987). Mutational analysis has shown that ARSs contain multi-domain DNA sequences. One of these domains, the A/T rich ARS Consensus Sequence (ACS), is conserved across all ARS elements. The ACS consists of 11 bp (Broach et al., 1983) and it is essential but not sufficient for ARS function (Celniker et al., 1984). The ACS together with an additional site 3’ to the ACS facilitate the binding of a multiprotein

complex termed the Origin Recognition Complex (ORC) to double stranded DNA (Bell and Stillman, 1992). ORC is therefore analogous to a multiprotein Replicon ‘initiator’.

The fission yeast *Schizosaccharomyces pombe* (*S. pombe*) is phylogenetically distant from *S. cerevisiae*. *S. pombe* also contain ARS-like elements capable of conferring autonomous replication in a transforming plasmid (Johnston and Barker, 1987). However, they do not contain specific sequences analogous to the 11 bp ACS, but consist of redundant A/T rich stretches of DNA (Maundrell et al., 1988, Clyne and Kelly, 1995) which recruit the *S. pombe* homologue of ORC (Ogawa et al., 1999).

The binding of ORC in *S. cerevisiae* appears to influence nucleosome positioning around the ACS, creating a more open chromatin environment, similar to open transcription promoters (Berbenetz et al., 2010, Eaton et al., 2010). In *S. pombe* the preference for A/T rich DNA may suggest that a relaxed DNA structure is important for origin selection. Taken together this data suggests that although origin selection has an intrinsic element that is controlled by the DNA sequence itself, there is an additional level of influence from the surrounding chromatin environment. With the evolution of defined origins of replication, both *S. cerevisiae* and *S. pombe* ensure that there is a sufficient number of replicators for each round of DNA replication and that origins are distributed to ensure DNA replication can be carried out efficiently with no sections of DNA left unreplicated (Newman et al., 2013).

1.3.3 Origins of Replication in Higher Eukaryotes

In higher eukaryotic cells, such as animal cells, origins of replication are ill-defined, and it appears that these cell types may lack the equivalent of a conserved Replicon ‘replicator’. To date no defined genetic element has been identified that determines an origin in metazoans. DNA of various sources replicate efficiently independently of DNA sequence and initiation occurs randomly in *Xenopus* egg extracts (Mechali and

Kearsey, 1984, Blow and Laskey, 1986, Hyrien and Mechali, 1992, Mahbubani et al., 1992) and human cells (Krysan and Calos, 1991). However whether conserved DNA sequences play a role in metazoan origins remains unresolved. This is due in part to the unsuccessful application of genetic assays that isolate replication origins in higher eukaryotes such as those that enabled a thorough characterisation of *S. cerevisiae* origins.

Significant progress has been made recently using sophisticated technologies such as DNA microarrays and genome-wide sequencing of nascent DNA strands during replication to map replication origins. However, a comparison of datasets generated using these techniques has revealed that the origin mapping data is discordant, with significant variation arising due to differences in the techniques applied and cell types that were used (Hyrien, 2015). In spite of this, some common features have been identified across numerous datasets and some origins of replication have correlated well with transcription start sites and CpG islands (Cadoret et al., 2008, Sequeira-Mendes et al., 2009, Cayrou et al., 2011, Valenzuela et al., 2011). There was also significant overlap in datasets suggesting that G-rich motifs associated with origins in *Drosophila*, mouse and human cells (Cayrou et al., 2011, Besnard et al., 2012, Cayrou et al., 2012).

While there is still some uncertainty about the role of the DNA sequence itself, there is undoubtedly a role for chromatin organisation and it appears that open chromatin states may aid in recruiting ORC. A transition from random initiation sites to intergenic sequences is observed when significant transcription occurs at the midblastula (MBT) transition in developing *Xenopus* (Hyrien et al., 1995) and *Drosophila* embryos (Sasaki et al., 1999). In addition, specific histone modifications particularly methylation and acetylation are associated with origin sites and may open up chromatin to facilitate origin selection (Sherstyuk et al., 2014). It is becoming apparent that there are multiple

regulatory mechanisms that influence the placement of origins in higher eukaryotes. Origin selection is influenced by cell type and state of differentiation. Therefore it is likely that multiple mechanisms have evolved to allow a more plastic placement of an origin and enable a metazoan cell to adapt to a more challenging lifecycle than those of single celled eukaryotes.

1.4 DNA Replication Occurs Once per Cell Cycle

1.4.1 Dealing with thousands of origins

Replicating massive eukaryotic genomes with high processivity and precision requires many origins of replication to fire within a single S-phase. This however raises another potential difficulty: if multiple origins fire at different times throughout S-phase how do cells differentiate between unreplicated DNA and the newly synthesised DNA strands? How do they ensure no origins fire more than once? Origin firing is coordinated to ensure DNA is replicated completely, with no section of DNA being replicated more than once. To achieve this, the cell must differentiate between replicated and unreplicated DNA.

1.4.2 Cell Fusion Experiments

Rao and Johnson carried out cell fusion experiments to investigate whether nuclei from different stages of the cell cycle could be induced to undergo DNA replication (Rao and Johnson, 1970). They observed that G1 cells fused to S-phase cells underwent DNA replication prematurely. This suggested that G1 nuclei were competent to respond to signals generated in S-phase cells that trigger DNA replication. However, G2 cells fused to S-phase cells did not rereplicate their DNA while the S-phase cells replicated DNA normally, suggesting G2 nuclei are not competent to respond to the DNA replication inducing signals of S-phase cells. When G2 cells were fused to G1 cells, G1 nuclei replicated normally, suggesting there was no inhibitory activity in the G2 cells

(Rao and Johnson, 1970). Taken together, these findings suggested that G1 cells are competent to respond to a diffusible replication promoting factor, this competence is lost in G2 nuclei that have already been replicated and after passing through mitosis the replication competence is restored. These experiments suggested that cells have a mechanism allowing them to differentiate between prereplicative and postreplicative DNA and ensure once per cycle replication.

1.4.3 *Xenopus laevis* Cell Free System

Further insights into the mechanisms ensuring once per cycle replication arose from research performed in *Xenopus laevis* eggs and egg extracts. *Xenopus* eggs are naturally arrested at metaphase II of meiosis and enter the cell cycle upon fertilisation, which is triggered by an intracellular wave of calcium. In only 7 hours the eggs rapidly carry out 11 rounds of DNA replication and cell division. During these rapid embryonic cell cycles there are negligible G1 or G2 phases and DNA replication lasts approximately 20 minutes *in vivo*. During the 12th cell cycle significant transcription begins triggering MBT and the cell cycles become elongated. These eggs carry out the first 11 cycles in the absence of transcription and maintain large stockpiles of mRNA and proteins that function during the cell cycle. DNA microinjected into these eggs replicates semi-conservatively and only once per cell cycle, demonstrating that this system can maintain normal cell cycle events (Harland and Laskey, 1980).

Due to the large size of the *Xenopus laevis* eggs, their stockpiles of replication proteins and their ability to regulate DNA replication similar to mammalian cells, they became an ideal source for an *in vitro* DNA replication system. A cell-free frog egg extract system was prepared to study the effect of purified cytoplasm on demembranated sperm chromatin (Lohka and Masui, 1983). This system was developed further and *Xenopus laevis* egg extracts were prepared that could support semiconservative semi-

discontinuous replication on a range of DNA templates with varying efficiencies (Blow and Laskey, 1986). Efficiency was in part dependent on the ability to form a nucleus (Blow and Laskey, 1986) with a bilayer and nuclear pores capable of importing protein using adenosine triphosphate (ATP) (Newmeyer et al., 1986). In egg extracts nuclei form around sperm DNA at different times and individual nuclei are able to replicate independently (Blow and Watson, 1987). This suggested that in addition to signals generated in the cytoplasm that promote S-phase, some intranuclear signal must regulate the initiation of thousands of origins at the level of the individual nucleus. Despite remaining in the S-phase promoting extract, individual nuclei never rereplicated their DNA suggesting that the nuclear environment may play a key role in the regulation of once per cycle replication. Therefore, *Xenopus* egg extracts represented an ideal system for the biochemical analysis of proteins involved in the initiation of eukaryotic DNA replication.

1.4.4 Licensing Factor Model

Blow and Laskey emulated the results of Rao and Johnson in *Xenopus* egg extracts. They showed that there is a single round of DNA replication per cell cycle and that G2 nuclei do not rereplicate when transferred into fresh extract. To replicate again nuclei must pass through mitosis during which time the nucleus breaks down. Rereplication could be induced in G2 by disruption of the nuclear envelope (Blow and Laskey, 1988). This suggested that DNA replication is controlled by compartmentalisation of replication factors between the nucleus and cytoplasm and led to the development of the licensing factor model. The licensing factor model predicted the existence of a factor which would establish once per cycle replication due to four definitive features:

1. It must bind to or modify DNA before S-phase.
2. It must be essential for the initiation of DNA replication.

3. It must support a single initiation event and must be removed or inactivated by DNA replication.
4. It must be unable to gain access to the nucleus until after the nuclear envelope breaks down in mitosis.

1.4.5 Evidence Supporting the Licensing Factor Model

Nuclear formation is a requirement for DNA replication in egg extracts (Newport, 1987, Blow and Sleeman, 1990) and in extracts treated with nuclear pore inhibitors the initiation but not elongation of DNA replication was inhibited (Cox, 1992). The role of the nucleus in ensuring once per cycle replication was confirmed when nuclei from synchronised HeLa cells were added to *Xenopus* extracts. G1 nuclei replicated semiconservatively whereas G2 nuclei were unable to rereplicate. However, permeabilisation of the G2 nuclei during their preparation resulted in replication upon addition to extracts. This showed that the nuclear membrane is crucial for distinguishing between pre- and post- replicative DNA and for the mechanisms that ensure once-per-cycle replication (Leno et al., 1992). These data fit the licensing model where permeabilisation of the membrane would allow the licensing factor access to the DNA to support more initiation events. It was also shown that the inability to replicate G2 nuclei is due to the absence of a positive acting factor rather than the presence of a negative replication inhibiting factor (Coverly et al., 1993).

The most significant evidence in support of the licensing factor model came from experiments that utilised a broad-spectrum kinase inhibitor, 6-(Dimethylamino)purine (6-DMAP). 6-DMAP treated metaphase extracts were unable to initiate DNA replication despite being able to form normal interphase nuclei and therefore lacked an activity that satisfied all 4 features of the licensing factor model:

1. A 15 minute preincubation in untreated activated extract was sufficient to allow DNA to be completely replicated in 6-DMAP extract, suggesting that during this

15 minute period the DNA had been modified in some way that did not require nuclear formation, as nuclear formation took 20 minutes in these extract. This ability was lost in 6-DMAP treated extracts. Therefore 6-DMAP extract lacked the ability to modify or 'license' chromatin itself.

2. 6-DMAP treated metaphase extracts failed to initiate DNA replication, but could form nuclei, a prerequisite for DNA replication and could elongate previously initiated replication forks. Therefore 6-DMAP extracts contained activities necessary for DNA replication but lacked a factor essential for the initiation of DNA replication.
3. G2 nuclei do not rereplicate when transferred to fresh extract, unless permeabilised. Permeabilised G2 nuclei did not rereplicate, however, when transferred to 6-DMAP extract. This shows that the activity inhibited by 6-DMAP supports a single initiation event.
4. Intact 6-DMAP nuclei transferred to untreated extract do not initiate DNA replication. Permeabilisation of the 6-DMAP nuclei however resulted in replication upon transfer to untreated extract. This shows that 6-DMAP nuclei lack a factor which cannot cross the nuclear envelope.

With data to support the existence of an essential licensing factor, an in depth biochemical characterisation of the initiation of chromosomal DNA replication in *Xenopus* was now required to determine what the licensing factor was and how it worked. Alternative approaches were performed in yeast to molecularly characterise the difference between G1 and G2 nuclei in *S. cerevisiae*. Genomic footprinting experiments were carried out using a DNase I protection assay to determine the nucleotide position of protein-DNA complexes. Early work on plasmids containing ARS sequences identified a protein binding extensively to the ACS which resembled the footprint of ORC (Bell and Stillman, 1992, Diffley and Cocker, 1992). It was then

shown that replication origins exist in distinct states pre- and post-replication. The post-replicative state, from S-phase to mitosis, resembled that obtained *in vitro* with mixtures of ORC and origin DNA, whereas the pre-replicative state formed upon mitotic exit and persisted throughout G1 and consisted of an extended footprint (Diffley et al., 1994). This suggested that additional factors bound at origins before S-phase and were removed from origins as replication initiated, consistent with the replication licensing model. This complex was denoted the pre-replicative complex (pre-RC)

1.5 DNA Replication Licensing

1.5.1 The MCM2-7 Complex is The Licensing Factor

The licensing factor was purified to homogeneity from *Xenopus* egg extracts and found to consist of heterohexameric minichromosome maintenance (MCM) complexes (Chong et al., 1997, Thömmes et al., 1997). The licensing reaction has since been shown to be molecularly defined as the loading of MCM2-7 complexes onto chromatin at origins of replication. The MCM genes were first identified in an *S. cerevisiae* screen where mutants in *MCM2*, *MCM3*, and *MCM5* failed to retain centromere- and ARS-containing minichromosomes over a number of cell cycles (Maine et al., 1984). *MCM4* (originally *CDC54*) and *MCM7* (originally *CDC47*) were isolated as cell division cycle mutants (Moir et al., 1982, Hennessy et al., 1991) and *MCM6* (originally *mis5*) was originally identified as a chromosome segregation mutant in *S. pombe* (Takahashi et al., 1994). To simplify the nomenclature these genes were renamed *MCM2* through *MCM7* (Chong et al., 1996). *MCM* genes are highly conserved among eukaryotes and are present in archaea. Each *MCM2-7* genes possesses unique sequences that define distinct evolutionarily conserved classes. Eukaryotic organisms have six genes, one from each class, as the MCMs are not functionally redundant, while archaea typically contain a single *MCM* gene. *MCM2-7* genes also possess regions with significant sequence homology and the proteins are members of the of AAA⁺ ATPase family.

MCM2-7 proteins form a 600 kDa heterohexameric ring-shaped complex in a 1:1:1:1:1:1 stoichiometry (Adachi et al., 1997, Fujita et al., 1997, Kubota et al., 1997).

MCM proteins were shown to be essential for the establishment of the prereplicative complex footprint in *S. cerevisiae* (Labib et al., 2001) and MCMs have been unequivocally shown to be the licensing factor hypothesised by Blow and Laskey (Figure 3). MCM function was heavily investigated after their initial discovery and after a decade of research it was apparent that the MCM2-7 complex satisfies each prediction of the licensing factor model:

1. MCMs bind to chromatin: MCMs bind to chromatin in yeast (Hennessy et al., 1990, Yan et al., 1993), *Xenopus* egg extracts (Chong et al., 1995, Kubota et al., 1995, Madine et al., 1995) and mammalian cells (Thommes et al., 1992, Hu et al., 1993, Todorov et al., 1994).
2. MCMs are essential for DNA replication: MCMs were shown to be essential for viability in *S. cerevisiae* (Hennessy et al., 1991) and *S. pombe* (Miyake et al., 1993, Takahashi et al., 1994) and function in the initiation of DNA replication at replication origins in *S. cerevisiae* (Sinha et al., 1986, Gibson et al., 1990, Yan et al., 1991, Chen et al., 1992, Yan et al., 1993, Dalton and Whitbread, 1995), *Xenopus* egg extracts (Chong et al., 1995) and mammalian cells (Kimura et al., 1994, Todorov et al., 1994, Fujita et al., 1996).
3. MCMs support a single initiation event at each origin. MCMs are removed from the DNA during S-phase in yeast cells (Hennessy et al., 1990, Hennessy and Botstein, 1991, Chen et al., 1992, Yan et al., 1993, Young and Tye, 1997), *Xenopus* egg extracts (Kubota et al., 1995, Chong et al., 1995) and mammalian cells (Kimura et al., 1994, Todorov et al., 1995, Krude et al., 1996). Following initiation, MCMs were found to associate with non-origin DNA, moving progressively away from origins (Aparicio et al., 1997).

4. MCMs are unable to bind to chromatin from late G1 until cells pass through mitosis. Although it is not excluded from the nucleus of mammalian cells, as the model predicts, MCMs are unable to bind or relicense chromatin until they pass through mitosis (Todorov et al., 1995) (Discussed in detail see section 1.7).

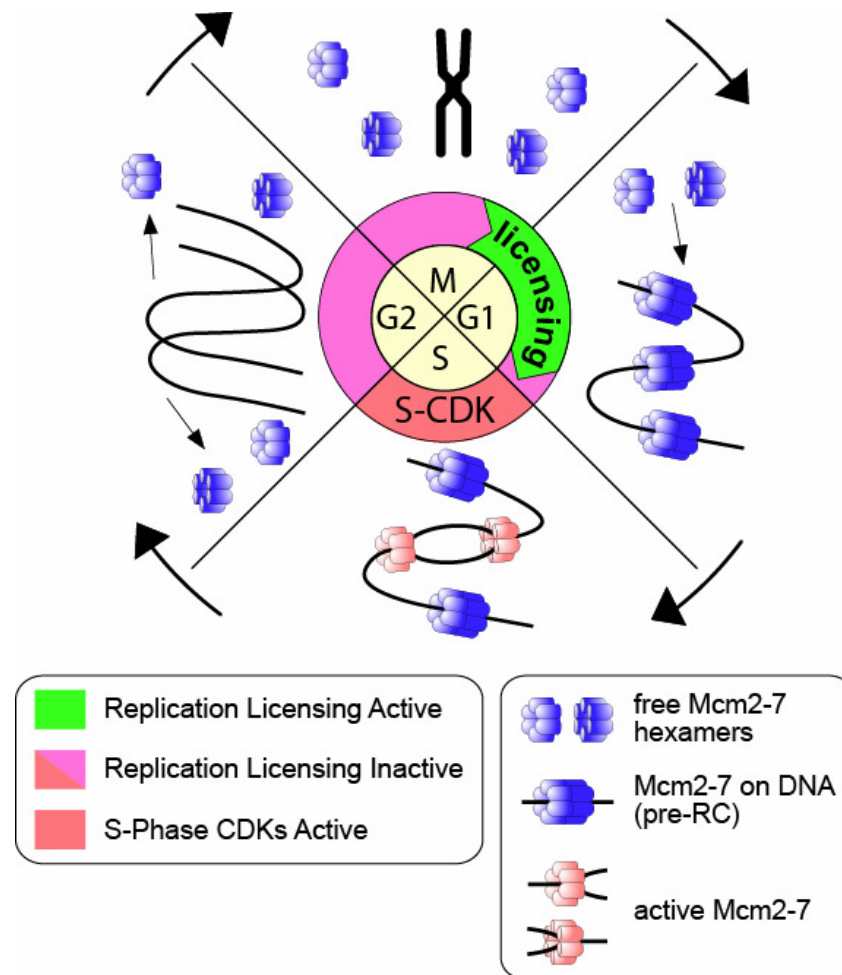


Figure 3: Regulation of Licensing during the Cell Cycle. DNA becomes licensed from late mitosis until G1 by the loading of MCM2-7 double hexamers. MCM2-7 hexamer activity removes MCM2-7 from the licensed origin during S-phase. Additional hexamers are unable to bind chromatin until cells pass mitosis and enter the next cell cycle.

Studies using *MCM* degron mutants suggested that MCMs were required for elongation in addition to initiation (Labib et al., 2000). In agreement with this, chromatin immunoprecipitation experiments have shown MCMs associate with non-origin DNA with a pattern similar to polymerase ϵ (Pol ϵ) during S-phase (Aparicio et al., 1997). Sequence comparison of conserved *MCM* motifs with DnaA, a prokaryotic ATPase

domain containing protein, identified walker A and walker B motifs, suggesting MCMs may be DNA-dependent ATPases required for DNA unwinding (Koonin, 1993). A purified dimeric heterotrimer of MCM4/6/7 was found to possess some weak 3'-5' helicase activity (Ishimi, 1997). Subsequent work has demonstrated that MCM2-7 represents the eukaryotic replicative helicase (Bochman and Schwacha, 2009, Riera et al., 2014). After loading inactive double hexamers onto DNA, cells enter S-phase where MCM2-7 is subjected to activating phosphorylations by Cdc7 and CDK activity leads to the recruitment of Cdc45 (Kubota et al., 2003) and GINS (Takayama et al., 2003). MCM2-7, Cdc45 and GINS together form a holoenzyme with highly processive DNA helicase activity termed the CMG (Gambus et al., 2006, Moyer et al., 2006, Ilves et al., 2010).

Metazoan MCM2-7 complexes in solution form a cracked ring structure and assume a left-handed lock-washer shape with a natural discontinuity called the MCM2/5 'gate' (Costa et al., 2011). This gap or gate allows for the entry and exit of double or single stranded DNA into the heterohexameric ring (Samel et al., 2014). After (pre-replicative complex) pre-RC assembly two hexamers form planarised conjoined rings (Evrin et al., 2009, Remus et al., 2009). After CMG formation and binding to single stranded DNA, ATPase domains form into a right handed spiral conformation (Costa et al., 2014). Therefore, distinct conformations are associated with the loading, activation and single stranded DNA binding of MCM2-7 suggesting that these conformations are required in the correct sequence for the complex to mature into a functional helicase. Cryo-electron microscopy (cryo-EM) data shows that double stranded DNA enters the MCM2-7 C-terminal AAA⁺ domains but the CMG binds to and translocates along single stranded DNA in the 3' to 5' direction (Costa et al., 2014) suggesting that DNA unwinding is carried out by a ploughshare mechanism (Takahashi et al., 2005). This model predicts

that a single hexamer translocates along duplex DNA and sterically separates the two DNA strands as they emerge from the helicase.

1.5.2 Reconstitution of Licensing: the essential factors

All the essential factors required for replication licensing, or the loading of MCM2-7 complexes onto chromatin, have been identified. There are three essential licensing factors: ORC, Cell division cycle 6 (Cdc6) and Cdc10-dependent transcript 1 (Cdt1). The licensing reaction has been reconstituted completely using proteins purified from *Xenopus* egg extracts (Gillespie et al., 2001) and later using tagged MCM2-7 complexes purified from *S. cerevisiae* cells arrested in G1 with recombinant ORC, Cdt1 and Cdc6 from bacteria or baculovirus (Evrin et al., 2009, Remus et al., 2009). These proteins are all highly conserved in eukaryotic cells. ORC, Cdc6 and MCM2-7 are part of the AAA⁺ class of ATPases. AAA⁺ proteins contain two domains, an ATP binding domain and a nucleotide interacting helix domain. ATP binding and hydrolysis coordinates the physical relationship between the two domains, allowing AAA⁺ proteins to convert the chemical energy provided by ATP hydrolysis into conformational changes that can exert a physical force on a protein complex. AAA⁺ proteins therefore have the ability to mediate the assembly and remodelling of protein complexes. This suggests that ORC, Cdc6 and Cdt1 act in concert to load MCM2-7 complexes onto the DNA by sequential ATP-coupled conformational changes. An introduction to each licensing factor is outlined below, and a detailed description of the molecular mechanism of MCM2-7 loading is outlined in section 1.5.3.

1.5.2.1. The Origin Recognition Complex

The Origin Recognition Complex, as previously noted, was identified as an ARS binding complex in *S. cerevisiae* (Bell and Stillman, 1992). It is a six subunit complex and proteins were named 1-6 in order from the largest to the smallest. Deletion of any of

these genes results in lethality in *S. cerevisiae* (Bell et al., 1993, Foss et al., 1993, Li and Herskowitz, 1993, Micklem et al., 1993, Bell et al., 1995, Loo et al., 1995). *ORC* genes are likely to exist in all eukaryotes. *ORC1-5* orthologues were identified in organisms as diverse as yeast, flies (Gossen et al., 1995), plants (Diaz-Trivino et al., 2005), frogs (Carpenter et al., 1996, Rowles et al., 1996, Romanowski et al., 1996) and humans (Gavin et al., 1995). ORC-like proteins are also found in most species of archaea (Barry and Bell, 2006). *ORC1-5* exhibit homology to AAA⁺ ATPases and each has a winged helix domain for DNA binding. Orc1, Orc4 and Orc5 contain functional ATP-binding sites in metazoans. *ORC6* genes are also present in metazoan genomes and are identified by an ORC6 protein fold. They have no homology to ATPases but resemble the general transcription factor IIB (Liu et al., 2011). ATP is required for the binding of ORC to DNA, however binding to DNA inhibits ORC ATPase activity (Bell and Stillman, 1992, Klemm et al., 1997).

ORC binding to origins serves as a scaffold for the sequential association of additional replication factors in all eukaryotes. This has been shown to be essential for initiation of DNA replication in *S. cerevisiae* (Fox et al., 1995), *S. pombe* (Grallert and Nurse, 1996) and *Drosophila* (Landis et al., 1997) cells while immunodepletion of ORC in *Xenopus* egg extracts abolishes replication (Carpenter et al., 1996, Rowles et al., 1996). The crystal structure of a eukaryotic ORC has recently been solved demonstrating that the complex resembles a bilayered ring where the winged-helix domains sit atop a layer of AAA⁺ folds (Bleichert et al., 2015). ORC proteins also have additional roles whereby they can influence nucleosome positioning, transcriptional silencing and they have been shown to interact with heterochromatin and centromeric DNA (Pak et al., 1997, Duncker et al., 2009, Eaton et al., 2010).

1.5.2.2 Cdc6

Cdc6 was first identified as a mutant in a set of cell division cycle screens in *S. cerevisiae* (Hartwell, 1976). Cdc6 is conserved in every eukaryotic organism and Cdc6 homologues are found in archaea (Barry and Bell, 2006). Cdc6 is an AAA⁺ family protein which has sequence homology to Orc1. Genetic studies in *S. cerevisiae* and *S. pombe* established a role for Cdc6 in the initiation but not elongation of DNA replication (Hogan and Koshland, 1992, Kelly et al., 1993, Nishitani and Nurse, 1995, Piatti et al., 1995, Muzi Falconi et al., 1996, Detweiler and Li, 1997). Biochemical studies in *Xenopus* egg extracts have shown that Cdc6 only binds chromatin in the presence of Orc2 and it is required for chromosomal DNA replication (Coleman et al., 1996). Similar observations were made in mammalian cells where microinjection of human Cdc6 antibodies blocked S-phase entry (Hateboer et al., 1998, Yan et al., 1998). Cdc6 is essential for pre-RC assembly in *S. cerevisiae* (Cocker et al., 1996, Santocanale and Diffley, 1996) and was shown to be essential for the chromatin binding of MCM2-7 in *Xenopus* egg extracts (Coleman et al., 1996) and *S. cerevisiae* (Aparicio et al., 1997, Donovan et al., 1997, Tanaka et al., 1997). MCM loading requires an intact Cdc6 AAA⁺ domain (Donovan et al., 1997, Perkins and Diffley, 1998, Wang et al., 1999, Schepers and Diffley, 2001).

Cdc6 also plays additional roles in the cell cycle, independent of its role in replication licensing. Cdc6 is a mitotic polo-like kinase 1 substrate and functions in regulating chromosomal segregation (Yim and Erikson, 2010). Cdc6 also interacts with ATR and is required for the ATR/Chk1 mediated S-phase checkpoint (Murakami et al., 2002, Oehlmann et al., 2004). Cdc6 can also interact with the polycomb protein Bmi1 and may play a role in transcriptional regulation (Agherbi et al., 2009).

1.5.2.3 Cdt1

Cdt1 was originally identified in *S. pombe* as an essential gene required for DNA replication that is under transcriptional control of Cdc10 (Hofmann and Beach, 1994). Cdt1 homologues have been identified in *S. cerevisiae* (Tanaka and Diffley, 2002b), *C. elegans* (Zhong et al., 2003), *Drosophila* (Whittaker et al., 2000), *Xenopus* (Maiorano et al., 2000) and humans (Wohlschlegel et al., 2000). It was subsequently shown to be a licensing factor required for chromatin loading of MCM2-7 in *S. pombe* (Nishitani et al., 2000), *Xenopus* egg extracts (Maiorano et al., 2000, Tada et al., 2001) and *S. cerevisiae* (Tanaka and Diffley, 2002b). Cdt1 contains two winged helix domains but no ATP binding motif unlike other replication licensing factors. The chromatin binding of Cdt1 is ORC and Cdc6 dependent (Maiorano et al., 2000, Gillespie et al., 2001). Cdt1 interacts with MCM2-7 via a C-terminal domain (Ferenbach et al., 2005). Cdt1 is a key target for regulation of replication licensing in metazoan cells (See Section 1.7). Cdt1 has an additional function in mitosis where it interacts with the Nec1 loop of the Ndc80 complex to facilitate stable microtubule kinetochore attachment (Varma et al., 2012).

1.5.3 Molecular Mechanism of Licensing

Licensing occurs by the stepwise assembly of ORC, Cdc6 and Cdt1 at origins and together they facilitate sequential loading of two MCM2-7 complexes onto DNA. The two MCM2-7 hexamers bind tightly together in a head-to-head orientation and remain inactive around double stranded DNA (Evrin et al., 2009, Remus et al., 2009, Gambus et al., 2011). Loading two MCM2-7 complexes head-to-head allows for the initiation of bidirectional forks in S-phase. Significant insights into the molecular mechanism of MCM2-7 chromatin loading have been gained from biochemical approaches and structural studies using reconstituted *S. cerevisiae* systems. More recently, single molecule analyses that can capture dynamic and transient events in real time have shed

light on the later steps in the reaction and have determined how the second MCM2-7 is recruited into the pre-RC.

ORC binds to origin DNA in an ATP-dependent manner (Bell and Stillman, 1992). An Orc4 catalytic arginine is essential to complete the ATPase active site of Orc1 (Bowers et al., 2004). DNA binding causes a conformational change, separating Orc1 and Orc4 (Sun et al., 2012) which suppresses ORC ATPase activity (Bell and Stillman, 1992, Klemm et al., 1997). This may prevent futile ATP hydrolysis before pre-RC assembly. ORC recruits Cdc6 to DNA. In *S. cerevisiae* Cdc6 plays a role in ensuring the correct placement of origins. Whereas ORC and Cdc6 form stable complexes at ARS sequences, non-origin DNA induces Cdc6 ATPase activity causing the complex to disassemble (Speck and Stillman, 2007). As metazoan origins may not require a genetic element for origin specification it is unclear whether Cdc6 plays a role as a specificity factor in these organisms. *S. cerevisiae* ORC/Cdc6 can bind and license non-origin DNA *in vitro* (Evrin et al., 2009, Remus et al., 2009), which is capable of initiating replication (On et al., 2014) suggesting it is formally possible that any DNA sequence can support licensing. However, Cdc6 has a fast off-rate and ORC/Cdc6 is a short-lived complex. ORC/Cdc6 at non-origin DNA has a half-life of 5.5 ± 0.1 s, whereas ORC/Cdc6 at ARS1 has a half-life of 9.6 ± 0.1 s giving a higher specificity to origins for MCM2-7 loading during *in vitro* reconstitution experiments (Duzdevich et al., 2015). Disassembly of the ORC/Cdc6 complex is likely due to ORC- and DNA-dependent Cdc6 ATPase activity (Randell et al., 2006b). Cdc6 binding induces a conformational change in ORC and the ORC/Cdc6 complex forms a ring shape that is likely to wrap around DNA (Sun et al., 2012, Sun et al., 2013). Cryo-EM data showed that in origin bound ORC/Cdc6 complexes Orc6 extends outwards to enable interactions with Cdt1 while the Orc4 arginine finger remains too distant from Orc1 and further

ORC conformational changes are therefore required to enable ATPase activity (Sun et al., 2012).

In *S. cerevisiae*, Cdt1 and MCM2-7 is imported into the nucleus as a complex (Tanaka and Diffley, 2002b) and Cdt1-bound MCM2-7 is recruited to DNA bound ORC/Cdc6. In metazoan cells however, Cdt1 and MCM2-7 bind independently to the origin. In the absence of Cdt1, a C-terminal Mcm6 extension blocks MCM2-7 binding to ORC/Cdc6 suggesting that Cdt1 binding causes conformational changes in MCM2-7 (Zhang et al., 2010, Evrin et al., 2013). The Mcm3 C-terminus interacts with Cdc6 during recruitment and mutants in this region block the interaction of Cdt1/MCM2-7 with ORC/Cdc6 (Frigola et al., 2013, Sun et al., 2013). It has been hypothesised that the licensing factors, behaving in a similar manner to that of a clamp loader, facilitate conformational changes to open the Mcm2 and Mcm5 'gate' and slide it over double stranded DNA (Yardimci and Walter, 2014).

In the presence of a non-hydrolysable ATP, an intermediate 1.1 MDa complex is formed consisting of ORC/Cdc6/Cdt1/MCM2-7 (OC₆C₁M) (Evrin et al., 2013, Sun et al., 2013). Cryo-EM data has suggested that the OC₆C₁M may wrap around DNA, as it was observed that a central channel traverses the entire length of the OC₆C₁M and may contain DNA (Sun et al., 2013). Confirming the MCM2-7 complex at this stage is wrapped around DNA, a rapamycin-inducible linkage between Mcm2/Mcm5 was shown to stabilise MCM2-7 on DNA during OC₆C₁M formation and before ATPase activity (Samel et al., 2014). In this 3 tiered complex the MCM2-7 AAA⁺ C-terminal domains interact with ORC/Cdc6 AAA⁺ domains and the MCM2-7 N-terminal domains face outwards. The MCM2-5 gate is partially open in this structure (Sun et al., 2013) and this complex is salt sensitive (Fernandez-Cid et al., 2013), indicating that it is not

stably bound around double stranded DNA. This may allow for quality control and release of MCM2-7 at this stage if required.

The OC₆C₁M is a very short lived complex (Sun et al., 2014) and its formation induces conformational changes in ORC/Cdc6, forming a spiral structure which leads to ORC/Cdc6 ATPase activity (Fernandez-Cid et al., 2013, Sun et al., 2013). The ATPase activity of Orc1 and Cdc6 triggers the release of Cdt1(Randell et al., 2006b)and the subsequent formation of an OC₆M complex (Fernandez-Cid et al., 2013). ATPase activity is dependent on the interaction of Cdt1 with Mcm6 and the OC₆M was shown to be a genuine precursor capable of recruiting a second Cdt1/MCM2-7 for the formation of a salt-stable double hexamer (Fernandez-Cid et al., 2013). OC₆M formation takes seconds in the presence of hydrolysable ATP, but conversion into salt-stable double hexamers was slow taking over 5 minutes. The C-terminus of Cdt1 was sufficient for OC₆M formation, but the N-terminus of Cdt1 was required for formation of salt-stable double hexamers (Fernandez-Cid et al., 2013). Differences in reaction kinetics and the requirement for independent Cdt1 interactions hinted that the recruitment of the first and second MCM2-7 complexes occur by different mechanisms. A further late-stage OC₆MM intermediate has been observed, however the quality of the cryo-EM made it difficult to determine the presence of Cdc6 and naming the complex OC₆MM was speculative, therefore this complex may have consisted of OMM alone (Sun et al., 2014). This indicated that MCM2-7 may dimerise on DNA before it is released from ORC.

Total internal reflection fluorescence microscopy of immobilised, fluorescently labelled DNA incubated with ATP and fluorescently labelled recombinant *S. cerevisiae* replication licensing factors has allowed for the study of single molecules in the licensing reaction (Figure 4). This technique enabled the recording

of protein arrival/departure times and short-lived intermediates were identified which could not be detected by the previously used structural and biochemical approaches (Ticau et al., 2015). This work confirmed that two MCM2-7 heterohexamers are recruited to the origin, one at a time. It was shown that Cdc6 always dissociates first from the OC₆C₁M, followed by Cdt1. This differs from previous models which suggested that Cdt1 was removed first (Fernandez-Cid et al., 2013). A second Cdc6 molecule was recruited to ORC followed by a second Cdt1/MCM2-7. Again, Cdc6 was found to dissociate first, followed by Cdt1 and ORC resulting in the loading of a double-hexamer of MCM2-7 (Ticau et al., 2015). It was found that a single ORC is responsible for recruitment of both MCM2-7 hexamers (Duzdevich et al., 2015, Ticau et al., 2015), ruling out numerous hypothesised models (Yardimci and Walter, 2014). Due to the physical separation, it is unlikely that ORC can interact directly with the second MCM2-7 hexamer and retention times of Cdc6 and Cdt1 are much longer during the loading of the second hexamer, correlating well with previous biochemical data that suggested the two hexamers are loaded by alternate different mechanisms. MCM2-7 complexes do not bind together in solution (Evrin et al., 2009), therefore it is likely that OC₆C₁M and OC₆M formation causes conformational changes in the MCM2-7 N-terminus, which allow it to interact with and load the second MCM2-7. In support of this, FRET-CoSMoS was used to determine the timing of double hexamer formation and it was shown the recruitment of the second MCM2-7 results in double hexamer formation prior to release of Cdc6 and Cdt1. This suggests that MCM2-7 N-terminal interactions occur before loading of the second hexamer. This is followed by Cdc6 release, the subsequent simultaneous release of Cdt1 and ORC and loading of a salt sensitive MCM2-7 head-to-head double hexamer (Ticau et al., 2015).

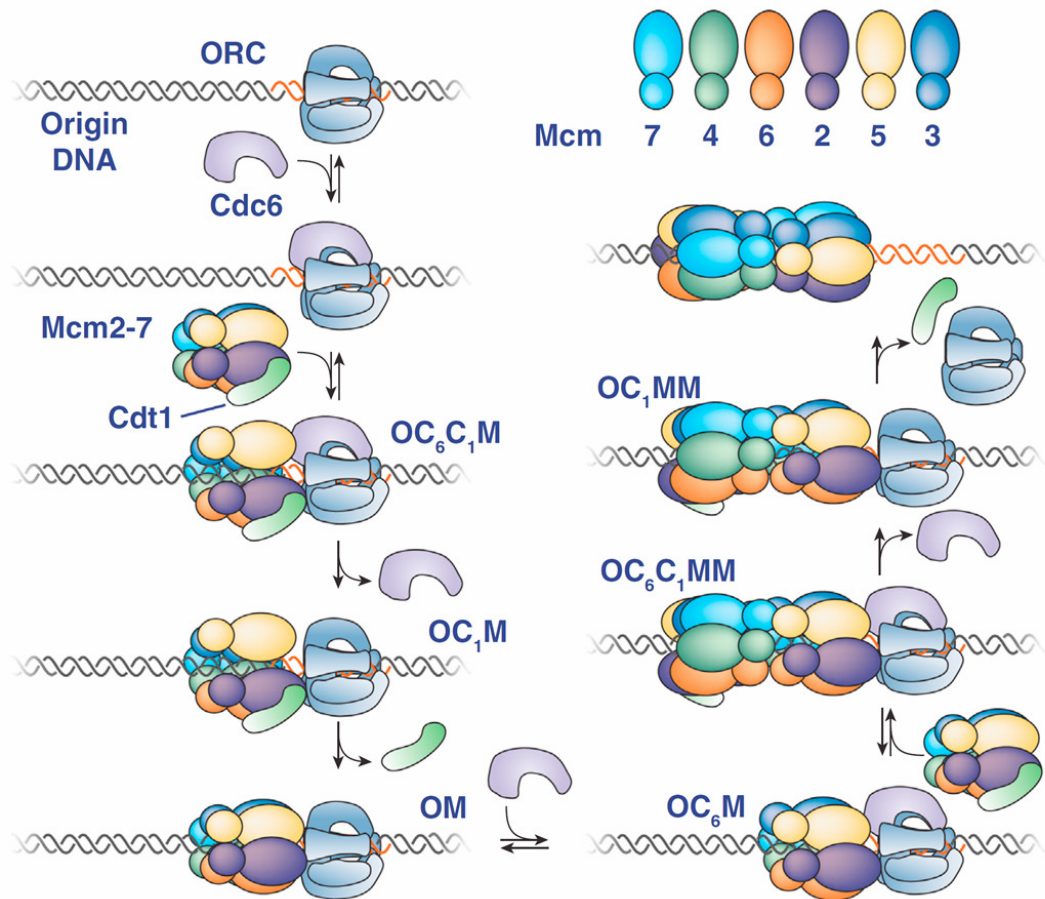


Figure 4: The Molecular Mechanism of Licensing. ORC binds to chromatin for the entire process of double hexamer formation. MCM2-7 complexes are recruited one at a time. ORC recruits Cdc6 and in turn Cdt1/MCM2-7 to form the OC_6C_1M . In the presence of hydrolysable ATP Cdc6 is released, followed by Cdt1. An additional Cdc6 is recruited, followed by Cdt1/MCM2-7, to form the OC_6C_1MM . Cdc6 is released first followed by Cdt1 and ORC, leaving a head-to-head double hexamer tightly wrapped around the DNA. Figure adapted from (Ticau et al., 2015). Further work is required to determine the ATP requirements of each step of the reaction.

1.5.4 Additional Factors that Effect Licensing

Reconstitution of licensing with *Xenopus* egg extract and *S. cerevisiae* purified proteins has identified the essential licensing factors, ORC, Cdc6 and Cdt1. Together with MCM2-7, DNA and ATP these are the minimal requirement for licensing *in vitro*. Additional factors have been identified that can influence licensing, however these are not functionally critical to the actual molecular mechanism of licensing, but rather facilitate licensing by other mechanisms or play regulatory roles.

1.5.4.1 Hbo1

Human acetylase binding to Orc1 (Hbo1) was identified as an Orc1 binding protein in a yeast two-hybrid screen from a HeLa cell cDNA library (Iizuka and Stillman, 1999). Hbo1 knockdown caused a significant reduction in MCM loading, but had no effect on ORC, Cdc6 or Cdt1 in human cells (Iizuka et al., 2006). Immunodepletion of Hbo1 in *Xenopus* egg extracts increased ORC and Cdc6 chromatin binding, reduced Cdt1 chromatin binding, blocked MCM loading and inhibited DNA replication. Replication was rescued by addition of Cdt1 (Iizuka et al., 2006). This suggested that Cdt1 was inhibited in Hbo1 depleted extracts. One caveat to this data however, was an issue with the specificity of the antibodies. Hbo1 is an approximately 70 kDa protein, whereas the depleted band in these extracts migrated at approximately 130 kDa on SDS-PAGE. Chromatin immunoprecipitation experiments in human cells demonstrated that Hbo1 can associate with origin DNA and this occurs specifically in G1 and depends on Cdt1. It was suggested that Hbo1 is a Cdt1 co-activator, as co-expression of Hbo1 in Cdt1 overexpressing cells triggered increased rereplication compared to overexpression of Cdt1 alone (Miotto and Struhl, 2008). Overexpression of Hbo1 and its co-factor Jade1 increased histone H4 acetylation at origins and enhanced MCM chromatin association. Inhibition of this H4 acetylation leads to a reduction in MCM loading, suggesting that H4 acetylation by Hbo1 at origins facilitates replication licensing (Miotto and Struhl, 2010). Taking advantage of an engineered CHO cell line that allows for microscopic visualisation of chromatin structural changes it was observed that targeting Cdt1 to a specific region resulted in a striking Hbo1- and H4 acetylation-dependent chromatin decondensation coupled with MCM loading in G1 (Wong et al., 2010). This indicates that rather than participating in the licensing reaction itself, Hbo1 may facilitate licensing by organising chromatin at the origin. Hbo1 may also represent a link between stress signalling and licensing. Activation of p53 in G1 leads to p53/Hbo1 interactions,

reduced Hbo1 histone acetyltransferase activity and reductions in MCM loading (Iizuka et al., 2008). In addition, JNK1 activation in response to stress leads to Cdt1 phosphorylation and reduction in Hbo1 recruitment to origins (Miotto and Struhl, 2011).

1.5.4.2 ORCA

ORC associated (ORCA) protein was identified by mass spectrometry as an ORC interacting protein and has been implicated to function in licensing. Tethering ORCA to an artificially generated LacO region resulted in ORC recruitment. Whether this resulted in licensing at this location was not tested (Shen et al., 2010). ORCA binds to chromatin in a cell cycle dependent manner and depletion of ORCA in human primary cells resulted in a reduction of ORC chromatin association, causing cells to accumulate in G1 (Shen et al., 2010). This suggests that ORCA aids in the stable association of ORC on chromatin. ORCA stability is Orc2 dependent and Orc2 knockdown results in ORCA destruction (Shen et al., 2010). ORCA has also been shown to interact with Cdt1, however the impact of this on licensing has not been investigated (Shen et al., 2012). Knockdown of ORCA resulted in a reduction of chromatin association of ORC. MCM2-7 chromatin binding was either unaffected or reduced depending on cell type (Shen et al., 2010). ORCA colocalises with ORC at heterochromatic sites and has been shown to bind to repressive histone marks, suggesting it may link licensing to higher order chromatin structure, or play a role in regulating the non-licensing functions of ORC (Bartke et al., 2010).

1.5.5 MCM Stability on Chromatin

A key property of MCM2-7 double hexamers that enables cell cycle regulation and ensures once per cycle regulation, is its ability to stably bind DNA once loaded. Once a double hexamer is loaded it remains indefinitely bound to chromatin until it is removed by DNA replication (Kuipers et al., 2011). This inherent ability of MCM2-7 double

hexamers to stably bind DNA is likely due to the conformational changes induced by their N-terminal interactions. In a head-to-head complex each MCM2-7 is twisted at a 30° angle, which is likely to inhibit ATPase activity and orientate the Mcm2-5 gates in two directions to prevent slippage off the DNA (Sun et al., 2014). Once an MCM2-7 double hexamer is formed it no longer requires any of the licensing factors. This allows for a sequential two-step reaction for the initiation of DNA replication that can be temporally separated by the cell cycle. Once MCMs have been loaded the licensing factors, ORC Cdc6 and Cdt1, are subjected to multiple forms of regulation to ensure that no licensing can occur after G1. Following licensing, in the next step towards initiation MCM2-7 complexes are subjected to S-phase specific phosphorylations which leads to the formation of an active helicase. CDK activity plays a key role in regulating the switch from the first to second step of initiation. As CDK activity rises during G1 it leads to the inactivation of licensing factors and once CDK activity reaches a threshold, cells enter S-phase where CDKs and other factors inhibit licensing while promoting replication initiation. This regulation is described in detail in section 1.7.

1.6 DNA Replication

1.6.1 Reconstitution of Initiation in *S. cerevisiae*

MCM2-7 chromatin loading is essential but insufficient for DNA replication. An activity termed S-Phase Promoting Factor (SPF) is required to activate licensed origins. SPF activity is provided by CDKs and Dbf4/Drf1 Dependent kinase (DDK). They act on multiple essential substrates to facilitate the assembly of a large pre-initiation complex (pre-IC) at a licensed origin. Pre-IC formation leads to the activation of the CMG and the subsequent unwinding of DNA. All DNA polymerases use a single stranded primer to synthesise a new DNA strand. RNA priming can only occur on single stranded DNA, therefore DNA unwinding is required prior to initiation. Significant work performed over the past 25 years has combined biochemical

investigations using *Xenopus* egg extracts and yeast genetics to identify and characterise proteins that function in the initiation of DNA replication. This has culminated in the *in vitro* reconstitution of replication initiation using 42 purified *S. cerevisiae* proteins, defining the minimal complement of proteins required for regulated replication initiation. For pre-IC formation; ORC, Cdc6, Cdt1, MCM2-7, DDK, CDK, Sld3, Sld7, Cdc45, Sld2, GINS, Dpb11 and Pol ϵ . For pre-IC activation; Mcm10, Pol α , RPA, Ctf4 and TopoII are required) (Yeeles et al., 2015)(Figure 5).

Salt stable MCM2-7 double-hexamers were loaded onto magnetic bead bound DNA using purified proteins. Licensed DNA was incubated with DDK and it was shown that MCM2-7 was the only essential DDK target *in vitro*(Yeeles et al., 2015). The exact sites remain unclear, but Mcm2, Mcm4 and Mcm6 appear to be important targets (Randell et al., 2010, On et al., 2014), however N-terminal mutants of Mcm5 can bypass DDK requirement (Hardy et al., 1997). There are two essential targets for S-phase CDK activity, Sld3 and Sld2. DDK-phosphorylated MCM2-7 is bound by Sld3/Sld7 and Cdc45. Sld2 phosphorylation facilitates interaction with Dbp11 in solution, leading to the assembly of an Sld2/Dbp11/GINS/Pol ϵ pre-loading complex (pre-LC) that is recruited to the chromatin bound Sld3/Sld7/Cdc45/MCM2-7 complex via interactions of Dpb11 with Sld3 (Yeeles et al., 2015). In this system the order of addition of CDK and DDK was not important, disagreeing with previous reports, which suggested that DDK must act first (Heller et al., 2011). However, yeast extracts were used in these experiments as opposed to purified proteins, and therefore they could have potentially contained phosphatases that would remove phosphorylations in the absence of ongoing DDK activity. DDK- and CDK-dependent recruitment of Sld3/Sld7/Cdc45 and Sld2/Dbp11/GINS/Pol ϵ leads to the formation of salt-stable CMG. MCM2-7 has been shown to encircle single stranded DNA in the CMG (Costa et al., 2014) and it is likely that formation of salt stable CMG in these assays correlates with extrusion of

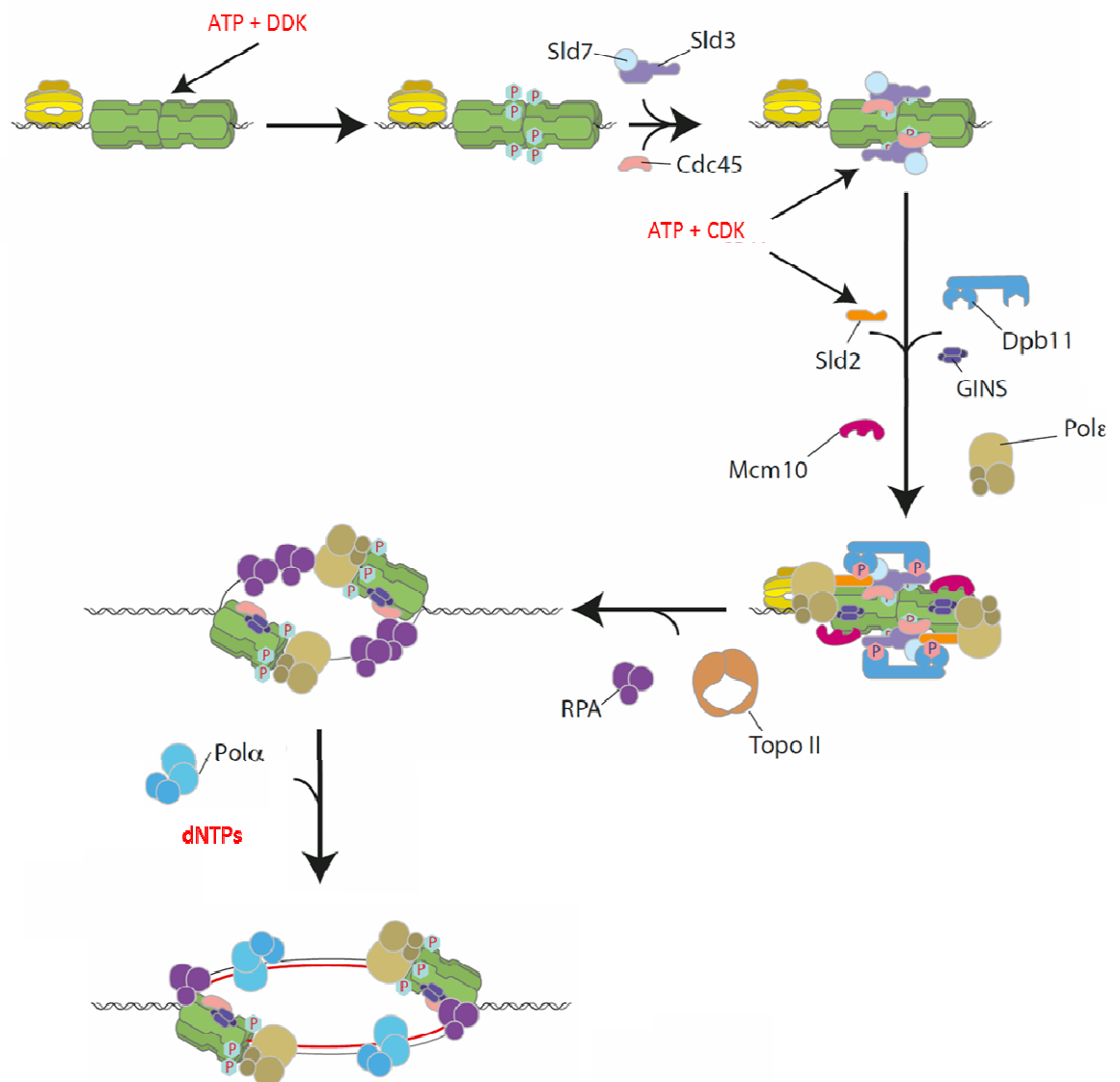


Figure 5: Reconstitution of the Initiation of DNA Replication in Eukaryotes. The combined action of DDK and CDK leads to the recruitment of Sld7/Sld3/Cdc45 and Sld2/Dpb11/GINS/Polε complexes to phosphorylated MCM2-7 double hexamers. Recruitment of MCM10 leads to the unwinding of DNA and additional factors, RPA and Topo II are recruited to coat single stranded DNA and unwind catenated DNA respectively. Recruitment of Polα leads to origin firing and dNTP incorporation. Figure was adapted from (Yeeles et al., 2015).

single stranded DNA. MCM10 is recruited next and this leads to the recruitment of RPA, indicating that the origin DNA has been melted due to CMG activation (Yeeles et al., 2015). A separate study demonstrated that RPA recruitment required ATP hydrolysis, suggesting that salt stable CMG formation may trigger MCM2-7

conformational changes leading to ATPase activation (Heller et al., 2011). Addition of Pol α allowed DNA replication to begin and triggered origin firing. RPA, TopoII and Ctf4 were required for efficient elongation, but were not absolutely required for origin firing. TopoII is required to relieve supercoils ahead of the replication fork and DNA catenation. Ctf4 links Pol ϵ to the CMG (Simon et al., 2014). Factors involved in DNA replication elongation (Pol δ , RFC, PCNA and Mrc1) were not used in this study and reconstitution of coupled leading and lagging strand replication will be a future goal for this system (Yeeles et al., 2015).

1.6.2 Metazoan pre-IC

There is significant conservation of the molecular machinery from yeast to mammals, with a few instances of functional orthologues, suggesting that the general mechanisms of initiation have been tightly conserved across eukaryotes. In metazoans, Treslin/Ticrr and TopBP1 are genuine homologs of Sld3 and Dpb11, respectively, and RecQ4 may be a functional orthologue of Sld2. However each of these three metazoan proteins have diverged significantly and have additional domains. CMG formation is also orchestrated by SPF in higher eukaryotes yet the essential CDK targets, analogous to *S.cerevisiae* Sld2 and Sld3 remain unknown.

Treslin contains a conserved Sld3 Cdc45-interacting site (Itou et al., 2014). CDKs phosphorylate Treslin in human cells and *Xenopus* egg extracts and this is required to promote the interaction of Treslin with TopBP1 and Cdc45, in addition to Cdc45 association with origins (Kumagai et al., 2010, Boos et al., 2011, Kumagai et al., 2011). MTBP has been proposed to be the functional homolog of Sld7, although it shares little to no sequence conservation with Sld7. However, MTBP interacts with Treslin throughout the cell cycle and its depletion prevents CMG assembly (Boos et al., 2013). RecQ4 has been suggested to be a Sld2 homolog despite very limited sequence

conservation. There is a weak similarity in a RecQ4 N-terminal domain that can interact with TopBP1. This domain is required for DNA replication in *Xenopus* egg extracts. However, RecQ4 is dispensable for Cdc45 and GINS recruitment, but is required for polymerase recruitment (Sangrithi et al., 2005, Matsuno et al., 2006). Therefore, whether or not RecQ4 is the functional homolog of Sld2 is still unclear. Human and *Xenopus* Mcm10 function differently than *S. cerevisiae* Mcm10 appearing to be required for CMG assembly rather than CMG activation (Wohlschlegel et al., 2002, Im et al., 2009, Di Perna et al., 2013). Additional novel factors with no homologues, GemC1 (Balestrini et al., 2010) and DUE-B (Chowdhury et al., 2010), have been implicated to have a role in Cdc45 recruitment, suggesting that helicase activation is more complex in higher Eukaryotes.

1.6.3 Replication Elongation

Once DNA unwinding is activated two separate forks move in opposite directions. The complex at each fork is referred to as the 'replisome' and all cells have the same core replisome components: helicase, DNA polymerases, circular sliding clamps, a pentameric clamp loader, primase and single strand binding protein (O'Donnell et al., 2013). Elongation of the first RNA primer becomes the leading strand and this is synthesised by Pol ϵ tethered to the CMG by Ctf4 (AND1 in human cells) (Simon et al., 2014). Multiple priming events occur on the lagging strand to form 100-200 bp Okazaki fragments synthesised by Pol δ . The RFC clamp loader assembles PCNA sliding clamps at the replisome to function with both Pol ϵ and Pol δ (Hedglin et al., 2013). DNA polymerases alone are distributive and synthesise few nucleotides before dissociation from DNA. PCNA essentially tethers the polymerases to DNA, substantially increasing processivity. A primase/polymerase switch mechanism couples leading and lagging strand synthesis making fork progression more processive.

1.6.4 Termination of Replication Forks

As two converging forks meet there must be a mechanism to ensure that the DNA between the two forks is replicated and the two replisomes become disassembled. Continuation of one fork and not the other would result in rereplication. It has recently been demonstrated in *S.cerevisiae* (Maric et al., 2014) and *Xenopus* egg extracts (Moreno et al., 2014) that Mcm7 becomes polyubiquitinated with K48-linked chains to facilitate CMG disassembly via Vcp/p97/Cdc48. This ubiquitination was carried out by the cullin family of ubiquitin ligases. This fits well with the replication licensing model that states that the licensing factor, MCM2-7 should be inactivated or removed by DNA replication.

1.7 Cell Cycle Regulation of Replication Licensing

1.7.1 Importance of Once Per Cycle DNA Replication

To accommodate for large genome sizes, eukaryotic cells initiate hundreds to thousands of origins of replication during S-phase. To ensure a complete yet once per cycle DNA replication and solve the problems associated with firing multiple origins, these cells use a two-step initiation reaction: licensing first, followed by origin firing. A key property of chromatin bound MCM2-7 double hexamers is their ability to remain stably bound for a number of hours in the absence of licensing factors. This allows for the sequential processes of licensing and origin firing to be temporally separated during the cell cycle. Thus, cells avoid the catastrophic effects of both under-replication and rereplication.

1.7.1.1 Consequences of Under-replication

In normal mammalian cells a p53 dependent licensing checkpoint exists to ensure cells cannot enter S-phase without a sufficient number of licensed origins (Nevis et al., 2009). Inhibition of licensing by expression of a non-regulatable geminin (Shreeram et

al., 2002) or knockdown of licensing factors (Feng et al., 2003, Machida et al., 2005, Liu et al., 2009, Nevis et al., 2009) causes cells to arrest in G1. This ‘licensing checkpoint’ ensures cells do not enter S-phase in conditions which would allow under-replication to occur. *S. cerevisiae* do not appear to have a licensing checkpoint and can enter S-phase in the presence of insufficient licensed origins. This leads to DNA double strand breaks, increased recombination and gross chromosomal rearrangements (Hogan and Koshland, 1992, Bruschi et al., 1995, Lengronne and Schwob, 2002, Tanaka and Diffley, 2002a). This kind of damage might lead to the development of cancer in multicellular organisms. Mouse models have been developed to study the effects of origin deficiency in mammalian cells. *Mcm4*^{chaos3} and *MCM2*^{IRE5-CreERT2} mutants result in reduced cellular levels of Mcm4 and Mcm2 respectively. This results in a consequent reduction in the level of licensed origins, leading to genomic instability and tumour development (Pruitt et al., 2007, Shima et al., 2007). A reduction in licensed origins increases inter-origin distance which leads to high levels of fork stalling (Kawabata et al., 2011). Replication intermediates were found to persist into mitosis, increasing the number of cells with lagging chromosomes, acentric fragments and micronuclei, likely the driving force behind the genomic instability and tumour development (Kunnev et al., 2010, Kawabata et al., 2011, Ozeri-Galai et al., 2011, Burrell et al., 2013). Overexpression of the oncogene cyclin E is another well studied example that demonstrates the detrimental effects of under-replication. Cyclin E is a G1/S cyclin and positive regulator of CDK2, which accelerates S-phase entry (Ekholm-Reed et al., 2004). This impairs MCM2-7 loading and cells enter S-phase with insufficient licensed origins (Ekholm-Reed et al., 2004). Overexpression of cyclin E and the consequent reduction of licensed origins is associated with longer replication tracks, increased ssDNA formation and RPA binding, increased fork stalling and genomic instability (Spruck et al., 1999, Bartkova et al., 2005, Bester et al., 2011). This data suggests that

under-replication of DNA due to deregulation of licensing can be a driving force for chromosomal instability and the development of cancer. Consistent with this, human cancer cells have compromised licensing checkpoints. These cells therefore enter S-phase in the presence of reduced licensed origins leading to DNA damage, abortive S-phase and apoptosis (Shreeram et al., 2002, Feng et al., 2003, Nevis et al., 2009).

1.7.1.2 Consequences of Rereplication

Licensing factors are subject to strict regulation during the cell cycle from late G1 until passage mitotic exit. No rereplication can occur during the periods of high CDK activity present from S-phase until mitosis. Rereplication causes genomic instability leading to and cell death in *S. cerevisiae* (Mimura et al., 2004, Wilmes et al., 2004) and mammalian cells (Zhu et al., 2004). How rereplication causes genomic instability is not well understood. In *S. cerevisiae*, *Xenopus* egg extracts and human cells rereplication causes DNA damage which leads to checkpoint activation (Vaziri et al., 2003, Melixetian et al., 2004, Zhu et al., 2004, Archambault et al., 2005, Green and Li, 2005, Li and Blow, 2005, Davidson et al., 2006, Zhu and Dutta, 2006, Liontos et al., 2007, Liu et al., 2007, Machida and Dutta, 2007). A number of mechanisms have been proposed to describe precisely how rereplication-dependent DNA breaks arise. At very high levels of relicensing, head-to-tail collisions of rereplicating forks were proposed to cause fork stalls that progress into DNA breaks in rereplicating *Xenopus* egg extracts (Davidson et al., 2006). In human cells it was shown that deregulated origin firing leads, by an unknown mechanism, to an accumulation of ssDNA gaps. This preceded double strand break formation and checkpoint activation. Persistence of ssDNA then caused double strand breaks when encountered by a rereplicating fork (Neelsen et al., 2013). Significant rereplication could deplete replication factors and dNTPs and impede fork progression, which could cause rereplication-induced ssDNA and DNA damage (Bester et al., 2011). The presence of incompletely replicated chromatids or unresolved repair

intermediates in mitosis can result in aberrant mitosis, generation of anaphase bridges, unbalanced chromosome segregation and subsequent genomic instability (Gisselsson et al., 2000, Janssen et al., 2011, Wilhelm et al., 2014). There is evidence to suggest that cancer cells do not possess an intact rereplication checkpoint (Vaziri et al., 2003, Liu et al., 2007), therefore deregulated licensing and rereplication could drive tumorigenesis. Consistent with this idea, licensing factors are frequently upregulated in cancer cells (Xouri et al., 2004, Tachibana et al., 2005, Tatsumi et al., 2006, Liontos et al., 2007, Jones et al., 2013). In addition, Cdt1 or Cdc6 overexpressing cells injected into transgenic mice led to tumour formation, demonstrating that Cdt1 and Cdc6 can both act as oncogenes, linking excessive licensing with cancer development (Arentson et al., 2002, Seo et al., 2005a, Liontos et al., 2007). However, Cdc6 oncogenic activity may be a consequence of a mechanism other than deregulated licensing, as Cdc6 overexpression was found to reduce transcription at the INK4/ARF locus (Gonzalez et al., 2006b).

1.7.2 Regulation of Licensing Factors in *S. Cerevisiae*

The control of replication licensing in yeast is mediated entirely by CDK activity. Each of the licensing factors, ORC, Cdc6 and Cdt1/MCM2-7 are subjected to control by CDK activity. CDKs regulate protein expression, subcellular localisation and activity. They also mediate the binding of inhibitors and targets proteins for destruction. The reliance of regulation on CDK activity has been demonstrated where overexpression of a CDK inhibitor, Sic1, permitted relicensing in S-phase and G2 with consequent rereplication (Dahmann et al., 1995, Noton and Diffley, 2000).

1.7.2.1 ORC

In *S.cerevisiae* Orc2 and Orc6 have multiple CDK phosphorylation sites. Hyperphosphorylated Orc2 and Orc6 are observed from late G1 until mitosis (Nguyen et al., 2001, Wilmes et al., 2004). Orc6 also contains a cyclin-binding (Cy) motif which

mediates an interaction with Clb5, the subunit for S-phase CDK (Wilmes et al., 2004). CDKs do not affect ORC ATPase activity, ORCs ability to bind to bead-bound origin-containing DNA or the recruitment of Cdc6 to ORC on DNA (Chen and Bell, 2011). In the presence of nonhydrolysable ATP, CDK-phosphorylated ORC can assemble the licensing intermediate OC₆C₁M complex but this complex cannot undergo changes required for OC₆M formation (Fernandez-Cid et al., 2013). Therefore, CDK inhibits the recruitment of the Cdt1/MCM2-7 complex to origin DNA by regulating ORC. Clb5 interaction with ORC can also sterically inhibit Cdt1 recruitment (Chen and Bell, 2011). Phosphorylation of Orc2 and Orc6 also blocks Cdt1 recruitment (Chen and Bell, 2011), although exactly how these phosphorylations regulate the molecular mechanism of licensing has not been investigated. Considering the intricacy of this molecular mechanism and the importance of sequential conformational changes it could be predicted that phosphorylation changes the structure of functional Orc2 and Orc6 regions. A complete ORC remains bound to *S.cerevisiae* chromatin throughout the cell cycle, where it likely plays additional roles in the cell cycle, thus a mechanism that can interrupt the molecular mechanism of licensing rather than the localisation or stability of ORC is required in this organism.

1.7.2.2. Cdc6

S. cerevisiae Cdc6 is subjected to multiple forms of regulation. CDK phosphorylation of the transcription factor Swi5 blocks its association with DNA (Moll et al., 1991) and this regulates Cdc6 expression, ensuring Cdc6 levels are high only in G1 (Zwerschke et al., 1994, Piatti et al., 1995). CDK phosphorylation of Cdc6 by G1 cyclins generates two distinct binding sites for the CRL1^{Cdc4} E3 ubiquitin ligase (Drury et al., 1997, Elsasser et al., 1999, Jang et al., 2001, Perkins et al., 2001). CRL1^{Cdc4} mediated ubiquitination leads to rapid degradation of Cdc6 during late G1 and S-phase (Drury et al., 1997, Drury et al., 2000). However, Cdc6 is more stable in late G2 and mitosis. This

is due to the mitotic cyclin Clb2, which binds to CDK phosphorylated Cdc6 and blocks one of the CRL1^{Cdc4} binding sites to increase G2/M stability of Cdc6 (Elsasser et al., 1996, Weinreich et al., 2001, Mimura et al., 2004). Clb2-bound phosphorylated Cdc6 is inactive and cannot bind to origin bound ORC to support MCM2-7 loading (Mimura et al., 2004).

1.7.2.3. Cdt1/MCM2-7

CDK phosphorylation of Mcm3 leads to the export of Cdt1/MCM2-7 from the nucleus from S-phase until cells pass through mitosis. Chromatin associated MCM2-7 is unaffected and remains bound until removal following DNA replication (Hennessy et al., 1990, Labib et al., 1999, Nguyen et al., 2000, Tanaka and Diffley, 2002b, Liku et al., 2005).

1.7.2.4 Redundancy and Rereplication

Inhibition of any single mechanism alone does not lead to significant levels of reinitiation in yeast (Drury et al., 1997, Labib et al., 1999, Nguyen et al., 2000, Nguyen et al., 2001, Wilmes et al., 2004). Mutating two pathways simultaneously generally leads to detectable rereplication and cell death; the severity of the phenotype very much depends on the combination of mutations (Wilmes et al., 2004, Mimura et al., 2004, Archambault et al., 2005, Green and Li, 2005). Loss of all three regulatory mechanisms, however, results in significant amounts of rereplication in G2/M-arrested cells (Nguyen et al., 2001, Chen and Bell, 2011) demonstrating the redundancy in the mechanisms yeast use to inhibit replication licensing. This redundancy is required to ensure the system is robust enough to prevent the detrimental effects of rereplication.

1.7.3 Regulation of Licensing Factors in Metazoans

In animals, reinitiation must be prevented at tens of thousands of origins during each cell cycle and over the course of development which involves billions of cell cycles.

Regulation of replication licensing in multicellular organisms therefore requires even more robust mechanisms to ensure once per cycle replication and metazoans have evolved additional control mechanisms that are not present in yeast. Replication licensing in animals is inhibited by three main mechanisms: inhibition of Cdt1, destruction of Cdt1 and regulation of pre-RC factors by CDKs. In these cell types, there is an apparent focus on Cdt1 regulation. Although CDKs do play a direct role in the regulation of replication licensing, downregulation of Cdt1 by multiple mechanisms is the major contributor to the inhibition of licensing (Fujita, 2006). Consistent with this, overexpression of Cdc6 or ORC individually does not cause significant rereplication in mammalian cells (Petersen et al., 1999, Vaziri et al., 2003, Saha et al., 2006, Tatsumi et al., 2006). However, in *Xenopus* egg extracts, *Drosophila* and mammalian cells, inhibition of Cdt1 regulation alone can induce significant rereplication, implying that mechanisms targeting ORC or Cdc6 are insufficient for preventing extensive rereplication (Vaziri et al., 2003, Melixetian et al., 2004, Nishitani et al., 2004, Thomer et al., 2004, Zhu et al., 2004, Arias and Walter, 2005, Li and Blow, 2005, Maiorano et al., 2005, Yoshida et al., 2005, Liu et al., 2007, Sugimoto et al., 2008). However, overexpression of both Cdt1 and Cdc6 has an even greater effect on rereplication than overexpression of Cdt1 alone (Vaziri et al., 2003).

1.7.3.1 Inhibition of Cdt1 by Geminin

Cdt1 activity is inhibited by geminin binding (Wohlschlegel et al., 2000, Tada et al., 2001) (Further discussed in Section 1.8). Cdt1 inhibition by geminin is the major pathway for the inhibition of licensing in *Xenopus* egg extracts (Tada et al., 2001, Li and Blow, 2005, Kisielewska and Blow, 2012). In mammalian cells geminin levels remain low in G1 due to APC/C activity and rise during late G1 to inhibit Cdt1 in S-phase, G2 and mitosis, providing a window of opportunity for licensing to occur during late mitosis and early G1 (McGarry and Kirschner, 1998, Wohlschlegel et al., 2000,

Nishitani et al., 2001). Geminin depletion or deletion induces a G2/M checkpoint, accompanied by substantial rereplication or S-phase delays depending on cell type (Klotz-Noack et al., 2012). These phenotypes are all most likely due to the induction of rereplication, highlighting the important role geminin plays in the regulation of replication licensing (Melixetian et al., 2004, Zhu et al., 2004, Karamitros et al., 2010). There is evidence that geminin may play a dual role in Cdt1 regulation by stabilising as well as inactivating Cdt1 in G2/M (Ballabeni et al., 2004, Ballabeni et al., 2013). This may ensure sufficient levels of Cdt1 are maintained for its mitotic function and also to ensure sufficient levels remain for efficient licensing after the metaphase-anaphase transition.

1.7.3.2 Inhibition of Cdt1 by Destruction

In human cells Cdt1 mRNA remains fairly constant throughout the cell cycle (Nishitani et al., 2001) but protein levels peak in G1 and decrease during S-phase (Wohlschlegel et al., 2000, Nishitani et al., 2001). In all eukaryotic cells Cdt1 is targeted for destruction by multiple mechanisms mediated either by PCNA, CRL1 or the APC/C. Cdt1 is targeted for destruction by the CRL4^{Cdt2} E3 ubiquitin ligase by a mechanism that is coupled to DNA replication (Kim and Kipreos, 2007, Abbas and Dutta, 2011, Havens and Walter, 2011). Cdt1 is recruited to CRL4^{Cdt2} via an interaction with PCNA during a normal S-phase (Zhong et al., 2003, Arias and Walter, 2006, Senga et al., 2006). This mechanism is conserved from fission yeast through metazoans (Arias and Walter, 2005, Jin et al., 2006, Ralph et al., 2006, Guarino et al., 2011) and PCNA-dependent ubiquitination is the main route for Cdt1 destruction during DNA replication and additionally to the DNA damage response (Higa et al., 2003, Arias and Walter, 2005, Arias and Walter, 2006, Jin et al., 2006, Lovejoy et al., 2006, Nishitani et al., 2006, Senga et al., 2006, Ishii et al., 2010, Roukos et al., 2011). How exactly Cdt1 is recruited to PCNA has not been investigated, although the interaction is known to be mediated by

a Cdt1 PCNA interacting protein box (PIP box) (Arias and Walter, 2006). Knockdown of components of CRL4^{Cdt2} E3 ubiquitin ligase has been shown to cause substantial Cdt1 dependent rereplication in *C. elegans*, Zebrafish and human cells (Zhong et al., 2003, Jin et al., 2006, Lovejoy et al., 2006, Sansam et al., 2006). During *Xenopus* early embryonic cell cycles however, a low nucleoplasm to cytoplasm ratio exists which allows Cdt1 levels to remain relatively stable (Kisieleska and Blow, 2012). Therefore this PCNA-coupled mechanism is likely to play a more significant role in cell cycles that are near to or post-MBT.

Cdt1 is also targeted for destruction by CRL1^{Skp2} in S-phase by cyclin A-CDK2 and (Li et al., 2003, Liu et al., 2004, Nishitani et al., 2004, Sugimoto et al., 2004, Takeda et al., 2005) in G2 by cyclin E-CDK2 (Liu et al., 2004). This is replication independent and requires CDK dependent phosphorylation of Cdt1 at threonine 29 (Liu et al., 2004). Importantly, however, inhibition of this mode of Cdt1 regulation by expression of a threonine to alanine mutant does not cause rereplication in human cells (Lovejoy et al., 2006).

In *Xenopus* egg extracts and human cells Cdt1 is also targeted for destruction by the APC/C after the metaphase to anaphase transition (Li and Blow, 2005, Sugimoto et al., 2008). This is thought to limit the accumulation of Cdt1 to prevent excessive licensing in G1 (Blow and Gillespie, 2008) and mutation of Cdt1 D-box resulted in rereplication in human cells (Sugimoto et al., 2008).

1.7.3.3 CDKs, ORC and Cdc6

A role for CDK regulation of preRC factors has been conserved in metazoans, however the specific outcomes of CDK phosphorylation tend to vary in different organisms. In *Xenopus* CDK targets Orc1 and Orc2 for phosphorylation (Carpenter et al., 1996). This results in the release of Orc1 from chromatin during S-phase (Rowles et al.,

1996) whereas the rest of ORC remains chromatin bound until it is released during mitosis by Orc2 phosphorylation (Hua and Newport, 1998, Findeisen et al., 1999, Rowles et al., 1999, Romanowski et al., 2000). In *Xenopus* egg extracts the inhibition of ORC plays a small but significant role in the prevention of relicensing in mitosis (Tada et al., 2001). In human cells Orc1 is removed from the DNA during S-phase until mitosis (Mendez and Stillman, 2000, Natale et al., 2000, Kreitz et al., 2001). S-phase CDKs phosphorylate Orc1 leading to CRL1^{Skp2} mediated destruction (Natale et al., 2000, Mendez et al., 2002, Ohta et al., 2003, DePamphilis, 2005). Phosphorylation and ubiquitination of Orc1 can also trigger nuclear export during S and G2 phase (Saha et al., 2006). Human Orc2 is also subjected to CDK dependent phosphorylation resulting in its dissociation from chromatin (Lee et al., 2012), consistent with observations in *Xenopus*.

In *Xenopus* Cdc6 is removed from chromatin after the binding of MCM2-7 and reloads in S-phase where it plays an important role in the regulation of the intra-S checkpoint (Oehlmann et al., 2004). In human cells CDK phosphorylation mediates export of ectopically overexpressed and endogenous Cdc6 to the cytoplasm beginning in late G1 lasting until mitosis (Saha et al., 1998, Fujita et al., 1999, Jiang et al., 1999, Petersen et al., 1999, Pelizon et al., 2000, Delmolino et al., 2001, Kim et al., 2007, Kim and Kipreos, 2008). Some Cdc6 remains bound to or rebinds chromatin (Fujita et al., 1999, Coverley et al., 2000, Mendez and Stillman, 2000, Alexandrow and Hamlin, 2004) where it plays a role in the S-phase checkpoint (Murakami et al., 2002, Clay-Farrace et al., 2003, Oehlmann et al., 2004). Human Cdc6 is also an APC/C^{Cdh1} target and levels fall quickly as G1 progresses (Mendez and Stillman, 2000, Petersen et al., 2000, Clijsters et al., 2013). Cdc6 also contains a PIP box, that mediates PCNA dependent destruction in S-phase to prevent Cdc6 reaccumulation (Clijsters and Wolthuis, 2014).

1.8 Geminin

1.8.1 Geminin Structure and Function

Geminin was first identified in a small pool expression screen as a protein that was degraded in mitotic *Xenopus* egg extracts (Lustig et al., 1997, McGarry and Kirschner, 1998). Geminin was shown to be a potent inhibitor of DNA replication, acting specifically at the licensing stage, as geminin addition to *Xenopus* egg extracts prevented MCM2-7 chromatin loading (McGarry and Kirschner, 1998). Geminin was found to inhibit licensing by inhibiting Cdt1 in *Xenopus* egg extracts and human cells (Wohlschlegel et al., 2000, Tada et al., 2001). Geminin homologues have since been found in worms, flies, avians, fish and mice (Quinn et al., 2001, Yanagi et al., 2002, Del Bene et al., 2004, Yanagi et al., 2005, Luo et al., 2007). No homologue has been found in yeast.

Geminin inhibits licensing by binding to Cdt1. Studies of the crystal structure of the coiled-coil of geminin alone or in a complex with Cdt1 by X-ray crystallography (Lee et al., 2004, Saxena et al., 2004, Thepaut et al., 2004, De Marco et al., 2009) and the analysis of geminin deletion mutants has defined several functional domains. The N-terminus is responsible for geminin destruction, nuclear localisation and also contains the geminin neutralising domain (described in section 1.8.3) (Figure 6). The coiled-coil of geminin consists of seven heptad repeats (*Xenopus* geminin 94-152) and mediates the formation of parallel homodimers. Dimerisation is mediated by multiple leucine and isoleucine residues (Saxena et al., 2004). Geminin forms an atypical coiled-coiled as there are several residues in the *a* and *d* positions of the heptad repeats which are not ideal for stabilisation (Saxena et al., 2004). It appears that geminin dimers can self-associate to form additional tertiary structures. Sedimentation velocity and sedimentation equilibrium analysis of full length *Xenopus* geminin identified a single

species with a molecular weight of 52.69 kDa, corresponding to a dimer (Benjamin et al., 2004).

Xenopus H	MNTNKKQRLD	MEKPTMSIKN	YFVDKTNESL	APRRTLKVIQ	PSASGCLVGR	50
Xenopus L	MNSNMKQRS	VENPSMSIKN	YIVDKTNEAL	APRRTLKVIQ	QSASGCLVGR	50
Human	MNPSMKQKQ-	-EEIKENIKN	-----SS	VPRRTLKMIQ	PSASGSLVGR	40
Mouse	MNLSMKQKQ-	-EGAQENVKN	-----SP	VPRRTLKMIQ	PSADGSLVGR	40
Xenopus H	TKEPVKNSTK	RKLWNDQLTS	KKAKVEVAVD	PEHRENKDC-	--SSEAYDLM	97
Xenopus L	TKEPAKNSTK	RKLWNDQLTS	KKAKVEVAVD	PEHQENKDC-	--PSEAYDLM	97
Human	ENELSAGLSK	RKHRNDHLTS	TTSSPGVIV-	PESSENKNLG	GVTQESFDLM	89
Mouse	ENELPKGLFK	RKLWDDQLAS	QTSSCG----	PEANENKDVG	DLTQEAFDLI	86
Xenopus H	VKETPTCLYW	KEVAEERRKA	LYEALQENEK	LHKEIELKDE	EIARLKQEND	147
Xenopus L	VKETPTCLYW	KDVAEERRKA	LYEALQENEK	LHQEIELKDE	EIARLKQEND	147
Human	IKENPSSQYW	KEVAEKRRKA	LYEALKENEK	LHKEIEQKDN	EIARLKKENK	139
Mouse	SKENPSSQYW	KEVAEQRRKA	LYEALKENEK	LHKEIEQKDS	EIARLRKENK	136
Xenopus H	ELMELAGHVQ	YMANMIERLT	GNAPRSLEDL	KDLDLEEARF	EDEADMAEAR	197
Xenopus L	ELMELAGHVQ	YMANMIERLT	GNAPQSLEDL	KNLDLEEARF	EDEA---ESR	194
Human	ELAEVAEHVQ	YMAELIERLN	G---EPLDNF	ESLDNQEFDS	EEET-VEDSL	185
Mouse	DLAEVAEHVQ	YMAEVIERLS	N---EPLDNF	ESPDSQEFDS	EEEE-VEYSE	182
Xenopus H	IEDE-----T	DMARPSNSDQ	NMDAHTV	219		
Xenopus L	IEDE-----T	DMTQFSSSDQ	NMDKQTV	216		
Human	VEDSEIGTCA	EGTVSSSTDA	---KPCI	209		
Mouse	LED SGAGTCA	EETVSSSTDA	---RPCT	206		

Figure 6: Geminin Structural Domains. Sequence alignment of *Xenopus laevis*, human and mouse geminin genes. Residues that control stability are in red. Residues that interact with Cdt1 are in blue. The neutralising domain is highlighted green. Areas implicated as having a role in nuclear import/export are highlighted purple. The coiled-coil domain is wrapped in black boxes. The Brg1 interaction domain is wrapped in an orange box.

SAXS data suggested that truncated coiled-coils form tetramers, or a head-to-tail dimer of dimers, in solution (Thepaut et al., 2004). The only structural work carried out the full length recombinant protein identified a human geminin tetramer, a parallel dimer of dimers, by cryo-EM (Okorokov et al., 2004). This structure required the N-terminal 80 amino acids for tetramerisation. This suggests that geminin exists as either a dimer or tetramer (dimer of dimers). Dimerisation of geminin is essential for Cdt1 inhibition (Benjamin et al., 2004, Saxena et al., 2004). Geminin-Cdt1 complex formation is mediated mainly by hydrophobic interactions. The N-terminal portion of

dimerisedgeminin coiled coils (*Xenopus* geminin 94-152) contains the primary Cdt1 interaction site. Mutation of this site causes a 2000-fold reduction in Cdt1 interaction as measured by isothermal calorimetry and mutants do not inhibit licensing in extracts (Benjamin et al., 2004, Lee et al., 2004, Saxena et al., 2004). A secondary interaction site is situated N-terminal to the coiled-coil (*Xenopus* geminin ⁹⁵DLM⁹⁷) and disruption of this interaction causes a 15-fold reduction in Cdt1 binding and licensing inhibition activity (Benjamin et al., 2004, Lee et al., 2004, Saxena et al., 2004). The identification of interaction sites, the determination of crystal structures and assays of geminin mutants together suggested that geminin binds Cdt1 to inhibit it, forming a heterotrimer.

A crystal structure of truncated human geminin:Cdt1 complexes was solved and indicated that geminin and Cdt1 can form a heterohexameric complex (De Marco et al., 2009) (Figure 7). Here, a tertiary interaction site at the C-terminus of the coiled-coil of geminin (Human geminin 145-160, *Xenopus* geminin 153-168) mediates an interaction between two head-to-tail geminin:Cdt1 heterotrimers. SAXS data supported the formation of a heterohexamer as truncated geminin:Cdt1 complexes formed a structure with a molecular mass of 90 kDa, matching the expected size, whereas deletion of residues 145-160 of geminin resulted in a reduction in complex size to ~37 kDa, as expected for a heterotrimer (De Marco et al., 2009). SAXS analysis of complexes consisting of full length Cdt1 and geminin indicated a molecular mass of ~165 kDa, suggesting that heterotrimer and heterohexamer formation may be a dynamic process (De Marco et al., 2009).

How exactly geminin binding inhibits Cdt1 is not fully understood. It has been proposed that steric inhibition of Cdt1 by geminin prevents interactions with MCM2-7 (Lee et al., 2004). Supporting this, it was shown that in the heterohexameric structure geminin

interaction buried key Cdt1 residues required for MCM2-7 loading (De Marco et al., 2009). In contrast to the licensing reaction described in yeast, Cdt1 and MCM2-7 are

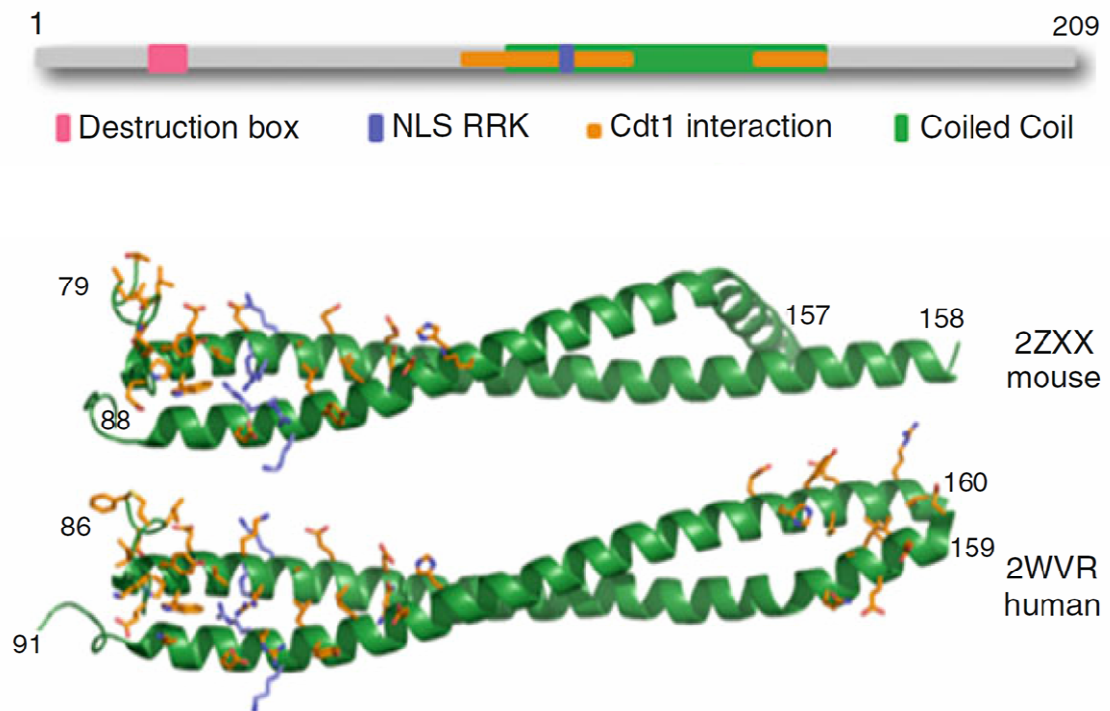


Figure 7: Crystal Structures of Geminin in Cdt1:Geminin Complexes. The crystal structure of geminin in a truncated mouse Cdt1:geminin complex (2ZXX) and a truncated human Cdt1:geminin complex (2WVR) is shown. Residues interacting with Cdt1 are highlighted in orange, NLS residues are highlighted in blue. The mouse geminin dimer makes two contacts with Cdt1 forming an axe-shaped heterotrimer. Human geminin dimers make two contacts with Cdt1 similar to the mouse structure with an additional interaction site at the C-terminus of a second Cdt1-bound geminin dimer, resulting in the formation of a heterohexamer. Figure adapted from (Caillat and Perrakis, 2012).

recruited separately to the origin in metazoans, potentially providing an opportunity for geminin recruitment and Cdt1 inhibition. Cdt1 can recruit geminin to chromatin suggesting that geminin inhibition of Cdt1 occurs at the origin (Gillespie et al., 2001, Xouri et al., 2007, Ode et al., 2011). In addition, it has been shown that addition of geminin or geminin^{DEL} (geminin lacking the destruction box (D-box)) in *Xenopus* licensing reactions results in the stabilisation of Cdc6 and Cdt1 on chromatin, similar to the addition of non-hydrolysable ATP in yeast assays (Gillespie et al., 2001, Randell et al., 2006a, Ode et al., 2011). This suggests that geminin may inhibit Cdt1-dependent

conformational changes required to trigger ATPase activity at the origin, either directly by binding Cdt1 or indirectly by preventing MCM2-7 recruitment. However, an alternative mechanism has been suggested, where geminin inhibits Cdt1's ability to bind to chromatin (Yanagi et al., 2002). Recent evidence suggests that this also occurs in *Xenopus* egg extracts. If geminin^{DEL} is added prior to DNA addition, Cdt1 is not recruited to DNA (Gillespie, P.J. unpublished data)

In somatic cells geminin is completely degraded by the APC/C^{Cdc20} at the metaphase-anaphase transition and is absent until transcription is triggered by E2F in late G1 (McGarry and Kirschner, 1998, Yoshida and Inoue, 2004, Clijsters et al., 2013). Geminin contains an N-terminal RXXL D-box motif that mediates its APC/C-dependent ubiquitination and subsequent degradation (Figure 6). Mutation of the D-box generates a non-regulatable geminin in *Xenopus* egg extracts and human cells (McGarry and Kirschner, 1998, Shreeram et al., 2002). Additional residues downstream of the D-box also contribute to geminin destruction. When the APC/C becomes active at the metaphase-anaphase transition it is able to selectively modify targets in a specific order, leading to a reproducible temporal pattern of target protein destruction that contributes to the tight control of cell cycle progression through mitosis. The E2 used by the APC/C, either Ube2E or UbcH10, influences the processivity of ubiquitination. In addition, there are intrinsic motifs on the substrate proteins, in addition to a D-box or KEN box, that influence processivity (Williamson et al., 2011). A motif originally identified in securin as the TEK box was shown to be required for the initiation of ubiquitination, independent of motifs required for APC/C interaction (Jin et al., 2008). This mechanism was investigated further and led to the identification of the ubiquitination initiation motif (IM) in geminin (Williamson et al., 2011). Differences in chain initiation motifs can determine the rate of substrate degradation. The geminin IM consists of ⁵⁰RTK-KRK⁶² and targets this protein for efficient and processive

ubiquitination making geminin an early target for destruction after the activation of the APC/C^{Cdc20} (Benjamin et al., 2004, Williamson et al., 2011)(Figure 6). Additional residues between the D-box and the IM have been implicated to play a role in geminin destruction in *Xenopus* egg extracts; mutation of ⁴²SASG⁴⁵ or ⁴⁷LVG⁴⁹ prevented destruction of recombinant geminin (Benjamin et al., 2004). Rapid geminin degradation allows Cdt1 to participate in the licensing reaction. This occurs in telophase immediately after APC/C activation (Dimitrova et al., 2002, Clijsters et al., 2013). High levels of geminin are then maintained from S-phase until mitosis, to ensure Cdt1 cannot initiate rereplication.

Geminin is a nuclear protein. *Xenopus* geminin has a nuclear localisation signal (NLS) and this motif is essential to prevent rereplication (Yoshida et al., 2005). Two bipartite NLS sequences have been reported, ⁷⁴RTK-KRK⁶² (Benjamin et al., 2004) and ⁶⁰KRK-KKAK⁷⁴ (Boos et al., 2006)(Figure 6). Data showing that a fluorophore labelled peptide of geminin 59-78 is imported into *Xenopus* nuclei lends supports to the ⁶⁰KRK-KKAK⁷⁴ motif as the NLS (Yoshida et al., 2005). However, the KKAK motif is only conserved in *Xenopus* and in mammalian cells a conserved RRK motif in the coiled-coil was shown to mediate NLS activity (Boos et al., 2006). The localisation of geminin RRK mutants was recovered by co-expression of Cdt1. This however suggests that geminin import in mammalian cells may not only be due to intrinsic signals, but also occur via Cdt1 interaction (Boos et al., 2006). *Xenopus* and mammalian geminin also contains a highly conserved nuclear export signal LEDLKDL and mutation of this motif can affect geminin localisation in mammalian cells (Boos et al., 2006, Luo et al., 2007)(Figure 6).

1.8.2 Geminin in Embryonic Cell Cycles

Although it was first identified as an unstable protein, it has been shown that 40-60% of geminin remains stable in interphase *Xenopus* extracts (Hodgson et al., 2002). The identification of geminin as a stable protein was initially a controversial observation as it was discordant with data from human cells and the fact that it was initially identified in a *Xenopus* egg extracts screen as an unstable protein. This disagreement was explained by an apparent difference in the regulation of endogenous and exogenously supplied recombinant geminin in the *Xenopus* extract, where recombinant protein is degraded with a much higher efficiency than the endogenous protein (Hodgson et al., 2002). In support of this, geminin was subsequently shown to be the main nuclear inhibitor of licensing in egg extracts (Hodgson et al., 2002, Arias and Walter, 2005, Yoshida et al., 2005, Li and Blow, 2005). Egg extracts do not support transcription therefore it is essential that geminin is not totally destroyed after cell cycle entry to ensure there is sufficient protein to prevent rereplication (Li and Blow, 2005).

The stabilisation of geminin is not a unique property of the *Xenopus* egg extract *in vitro* system. Geminin is stable *in vivo* in the embryonic cell cycles of a number of organisms. In the rapidly proliferating cells of the *Xenopus* early embryo the levels of Cdt1 and geminin are persistently high remaining stable the cell cycle, as observed by western blotting of embryo lysates (Kisielewska and Blow, 2012). In the *Drosophila* early embryo the first fourteen cell cycles occur in a syncytium within a single multinucleate cell, and consist of very rapid S-M cycles. Immunofluorescence microscopy of geminin in *Drosophila* embryos showed that geminin levels remain high during syncytial divisions, irrespective of cell cycle stage and show no evidence of mitotic destruction (Quinn et al., 2001). This implies that these cells can license and replicate DNA in the presence of geminin. Geminin was also present in the nucleus of endoreduplicating adult *Drosophila* ovaries which undergo multiple rounds of DNA

replication without passing through mitosis, again suggesting that DNA replication can occur in the presence of geminin in these specific cell types (Quinn et al., 2001). It was observed by immunofluorescence microscopy that all cells are geminin positive in an asynchronous culture of highly proliferative chick embryonic fibroblasts (Luo et al., 2007). Western blot analysis of synchronised CCE28 mouse embryonic stem cells showed that geminin is stable throughout the cell cycle including G1 (Fujii-Yamamoto et al., 2005). Consistent with this, geminin levels measured by western blot remained constant in mouse ES cells after a release from mitotic shake off. Importantly this did not affect entry into S-phase or inhibit DNA replication as indicated by BrdU incorporation (Yang et al., 2011). Taken together this data suggests that the stabilisation of geminin in G1 may be a common feature of rapidly replicating embryonic cell cycles.

1.8.3 Requirement for G1 Geminin

As described above, it appears that geminin is a stable protein during embryonic cell cycles. This creates a significant problem of how do cells proliferate in the presence of such a potent DNA replication inhibitor? How do these cells carry out licensing with stable levels of geminin throughout the cell cycle and why do these cells not destroy geminin? In fact it is essential that these cells do not destroy geminin. Not only is it likely that there is insufficient time to synthesise geminin prior to S-phase during fast cleavage cycles, but geminin has additional roles other than replication inhibition. Therefore it is possible that these cells have a mechanism to stabilise geminin during these cell cycles. Geminin was discovered in two separate small pool screens simultaneously in the Kirschner laboratory, one as a mitotically degraded replication inhibitor (McGarry and Kirschner, 1998) and another as a clone isolated from stage 6-7 blastulae that induces neural plate expansion post injection into embryos at the four cell stage (Kroll et al., 1998). *Xenopus laevis* is a pseudotetraploid organism with many functionally redundant genes with and closely related paralogues. Both *Xenopus* geminin

clones identified are 89% identical at the amino acid level and have identical properties. The Latin for twins is *gemi* and these proteins were therefore named geminin. Geminin has since demonstrated dual functionality where it has been proposed that to coordinates proliferation and differentiation decisions. By competitively interacting with transcription factors and chromatin remodelling complexes geminin regulates multiple pathways in a cell context dependent manner to influence development, organogenesis and cellular homeostasis.

During development geminin promotes the specification of pluripotent progenitors into an early neural lineage. In *Xenopus*, geminin's role in early neural lineage specification was first shown to be due to its ability to suppress BMP4 expression and upregulate proneural gene expression. This required a minimum region of geminin 38-90, designated the neutralising domain (Kroll et al., 1998). The molecular mechanism of geminin's role in neural specification is not understood; residues 38-90 do not include the coiled-coil which has been implicated in regulating numerous protein-protein interactions and it is likely that an unidentified interacting protein binds to geminin in this region. However, full-length geminin-dependent regulation of the epigenetic state has been demonstrated in *Xenopus* and mammalian cells and this could account for fate determination. By mediating the function of the Polycomb repressor complex and facilitating hyperacetylation of chromatin, geminin induces neuroectodermal specification and while restricting mesoderm, endoderm and ectoderm commitment by setting a threshold in cells that must be overcome for extrinsic stimuli to induce these alternative lineage fates (Lim et al., 2011, Yellajoshiyula et al., 2011, Yellajoshiyula et al., 2012, Caronna et al., 2013). Some insights into the molecular mechanism by which geminin carries out these functions have been gained from work in chick embryos. The interaction of geminin with Brm (Brg1 homolog), the catalytic subunit of the SWI/SNF complex, enhances Sox2 expression, the earliest marker for neural plate development.

This interaction is inhibited by the binding of ERNI binding to geminin. ERNI recruits HP1 to enhancer sequences on chromatin to repress Sox2 expression. Antagonistic interactions between BERT and ERNI facilitate geminin-dependent Sox2 expression. (Papanayotou et al., 2008).

After the initial lineage specification, geminin maintains neural progenitors in a proliferative state prior to subsequent differentiation. In *Xenopus*, geminin competes with proneural b-HLH transcription factors for the binding of Brg1 to inhibit transcriptional activity and control the timing of neurogenesis (Seo et al., 2005b, Yellajoshiyula et al., 2012). In contrast to this geminin inhibits the expression of neural genes in non-neural cells in the developing mouse, highlighting the importance of cellular context in determining the outcome of geminin activity during development (Kim et al., 2006).

Geminin has been implicated to function in various forms of organogenesis. In Medaka fish, geminin binds the homeodomain protein Six3 to antagonise the differentiation inducing role of Six3 during eye development (Del Bene et al., 2004). During mouse and chick development geminin also inhibits Hox family members through the Polycomb complex to regulate axial patterning establishment and body segmentation (Luo et al., 2004). Geminin also promotes cellular homeostasis in a number of contexts. As described above geminin maintains a pool of neural progenitors to control the timing of neurogenesis. In mouse and human cells, geminin is also involved in the maintenance of long term proliferating hematopoietic stem cells (Ohtsubo et al., 2008) and peripheral T-cells (Karamitros et al., 2010).

The strongest evidence supporting an essential role for geminin stabilisation in embryonic cell cycles, is the role of geminin in maintaining pluripotency. Geminin is required at the earliest stages of development during the cleavage cycles that occur

immediately after fertilisation. This is in contrast to cell context dependent fate-decision functions which occur later in development. Geminin-null mice embryos are pre-implantation lethal. Geminin knockout ES cells lose pluripotency, endoreduplicate and arrest at the 8-cell stage (Gonzalez et al., 2006a, Hara et al., 2006). Geminin antagonises Brg1 in these cells to maintain the expression of factors that establish pluripotency. Loss of geminin causes a loss of Oct4, Sox2 and Nanog, and these cells differentiate to trophoblasts (Yang et al., 2011). Interestingly, geminin is also required for the reprogramming of fibroblasts and the generation of induced pluripotent stem cells (Tabrizi et al., 2013), indicating that elucidating the mechanisms mediating the stabilisation of geminin in G1 could have an application in generating efficient iPS cells. Regulation of G1 length is a major determinant of differentiation. It is thought that a longer G1 allows transcription factors to accumulate and reach thresholds to commit cells to specific fate decisions. This combined with the fact that geminin maintains pluripotency suggests that it is essential that geminin is stabilised throughout G1 of embryonic cell cycles.

1.8.4 The Inactivation of Geminin

Injection of geminin mRNA into *Xenopus* embryos at the two-cell stage had no effect on proliferation in cells until after MBT (Kisieleska and Blow, 2012). As discussed above these cells maintain relatively stable levels of geminin. This suggests that even the addition of excess of geminin does not affect the cleavage cycles in the embryo, and that these cells are able to maintain proliferative capacity in the presence of constitutively high levels geminin (Kisieleska and Blow, 2012). Knockdown of geminin to ~20% of normal levels in mouse ES cells has no effect on ploidy or neural fate commitment. Despite such a large reduction in protein levels geminin maintains its normal functions, indicating that in untreated cells geminin is present in excess, yet these cells continue to divide rapidly (Yellajoshiyula et al., 2011). Together,

this evidence suggests that highly proliferative embryonic stem cells maintain high levels of geminin throughout the cell cycle to maintain pluripotency. This is somewhat paradoxical as geminin is a potent replication inhibitor. Therefore it is essential that these cells support mechanisms to inactivate geminin after mitosis to facilitate licensing and ensure proliferative capacity for the next cell cycle. Biochemical data gathered in *Xenopus* egg extracts lends support to this hypothesis and has provided evidence that geminin is indeed inactivated after the metaphase-anaphase transition.

Gel filtration of extracts showed that high molecular weight geminin-Cdt1 complexes break apart after the metaphase-anaphase transition (Hodgson et al., 2002). Recombinant geminin^{DEL} added to interphase extracts binds to Cdt1, reforming the high molecular weight complex, demonstrating that endogenous interphase Cdt1 is capable of interacting with geminin (Li and Blow, 2004). However, recombinant Cdt1 pulldowns from extracts demonstrated that interphase geminin only weakly interacts with Cdt1 (Hodgson et al., 2002). Taken together this suggests that endogenous interphase geminin has been inactivated and is no longer capable of binding Cdt1. In addition, geminin-Cdt1 complex reformation was observed following geminin nuclear import (Hodgson et al., 2002). This supports the idea that geminin activity is tightly regulated in egg extracts to facilitate licensing after the metaphase-anaphase transition and to inhibit rereplication in S-phase.

Several mechanisms have been hypothesised to describe geminin inactivation. There is evidence to suggest that geminin is regulated by post translational modification (PTM), by protein-protein interactions, or by regulation of gem-Cdt1 complex stoichiometry (Li and Blow, 2004, Hodgson et al., 2002, De Marco et al., 2009, Lutzmann et al., 2006, Ode et al., 2011).

The post-translational modification hypothesis fits well into a model where geminin is modified at the metaphase-anaphase transition rendering it inactive in the cytoplasm to permit Cdt1 activity. Nuclear formation and geminin import would then separate geminin from the modifying enzyme or increase the local concentration of demodifying enzymes in the nucleus in order to reactivate geminin and prevent rereplication. It has been shown that CDK-dependent APC/C activity is required for geminin inactivation and the activation of licensing (Li and Blow, 2004). However, proteasomal degradation was not necessary for the activation of licensing, suggesting that APC/C mediated ubiquitination can also control target protein activity by mechanisms other than proteasomal destruction (Li and Blow, 2004). This is true for cyclin B which can be inactivated by mono- or poly-ubiquitination (Dimova et al., 2012). Gel filtration analysis of metaphase extracts that were supplemented with very low amounts of S-labelled recombinant geminin or geminin^{DEL} prior to release into interphase demonstrated that the D-box motif was essential for the inactivation of geminin, leading the authors to conclude that APC/C mediated ubiquitination is required for geminin inactivation (Li and Blow, 2004). As inactivated geminin does not show a shift on SDS-PAGE indicative of ubiquitination, Li and Blow concluded that APC/C-mediated polyubiquitination of geminin may lead to a second modification.

An alternative hypothesis is that geminin is inactivated by interaction with other cell cycle regulated proteins. Geminin has been shown to interact with multiple coiled-coil proteins and that this is important for cell fate decisions. Pre-incubation of recombinant geminin with recombinant HoxA11 caused a reduction in geminin-dependent Cdt1 pulldown from cell extracts, suggesting HoxA11 competes with Cdt1 for geminin binding (Luo et al., 2004). Furthermore, overexpression of HoxB7 or HoxA11 in mouse embryonic fibroblasts reduced the amount of Cdt1 that co-immunoprecipitates with geminin (Luo et al., 2004). High levels of *Xenopus* Six3 or Six6 could compete with

recombinant Cdt1 for binding to GST-geminin *in vitro* (Del Bene et al., 2004), however, the Six6 interaction site was found to be distinct from the Cdt1 interaction sites. However, these specific interactions are unlikely to account for geminin inactivation during cleavage cycles as these transcription factors are not expressed until later in development. A number of laboratories have undertaken bioinformatic searches to identify novel replication factors by searching for sequence similarities to key domains of known replication proteins. Using the geminin coiled-coil as a query led to the identification of two proteins with geminin-like coiled-coils, Idas and GemC1, defining a new family of geminin-like proteins (Balestrini et al., 2010, Pefani et al., 2011). As the geminin coiled-coil mediates dimerisation and is required for the inhibition of licensing, these proteins are likely to interact with geminin due to the similarity in the coiled-coil regions and could therefore potentially represent novel geminin inhibitor proteins. The human geminin coiled-coil shares 53% identity with Idas at the amino acid level. Idas can interact with geminin in human cells and may carry out functions similar to geminin as it appears to participate in both the cell cycle and cell fate decisions. Depletion of Idas in human cells causes an abnormal S-phase, whereas overexpression results in multipolar spindle formation (Pefani et al., 2011). Idas was found to be a key regulator of multicilliate cell differentiation by coordinating cell cycle exit, centriole assembly and controlling the transcription of FoxJ1 (Stubbs et al., 2012). *Xenopus* GemC1 (Geminin coiled-coil containing protein 1) shares a 39% identity with the coiled-coil of human geminin at the amino acid level. It was shown that this protein cannot interact with Cdt1, however an interaction with geminin was not investigated. GemC1 was proposed to play a role in the initiation of DNA replication, specifically in the recruitment of Cdc45 to aid in CMG formation (Balestrini et al., 2010). A recent publication has identified a crystal structure of truncated human geminin:GemC1 heterodimers and demonstrated that these proteins can interact when expressed in

bacteria and human tissue culture cells, suggesting that GemC1 could play a role in regulating geminin function (Caillat et al., 2015).

There is evidence that geminin activity is controlled by the stoichiometry of the geminin:Cdt1 complex. It was originally thought that a geminin dimer binds to Cdt1 to form an inhibitory heterotrimer (Benjamin et al., 2004, Lee et al., 2004, Saxena et al., 2004, Ferenbach et al., 2005). Inactivation of geminin then releases Cdt1 to allow licensing to occur. This view was challenged after the identification of a heterohexameric geminin:Cdt1 complex which led to the hypothesis that heterotrimer is permissive for licensing and heterohexamers are not permissive for licensing (De Marco et al., 2009). An equilibrium between heterohexamer and heterotrimer could theoretically coordinate a switch to regulate licensing. This is an interesting hypothesis, as regulation of the relative levels of these proteins alone could regulate the activation of licensing. Additionally, heterotrimer and heterohexamer formation could be mediated by PTMs or protein conformational changes. There is biochemical evidence to support the existence of a permissive complex; a co-expressed and purified geminin^{DEL}-Cdt1 complex had licensing activity similar to Cdt1 alone when added to interphase extracts (Lutzmann et al., 2006).

1.9 Aim of this Thesis

Embryonic cell cycles proceed and DNA replication licensing occurs in the presence of constitutively high levels of the replication inhibitor protein geminin. Evidence from *Xenopus* egg extracts suggest that geminin is inactivated to allow licensing after the metaphase-anaphase transition and reactivated to prevent rereplication after nuclear formation. The aim of this project was to investigate the multiple hypotheses that have been proposed to regulate geminin inactivation and determine the molecular mechanism that accounts for the stabilisation and inactivation of geminin in *Xenopus* egg extracts.

2.0 Materials and Methods

2.1 Reagents and Standard Solutions

Reagents and standard solutions were made up in MilliQ in H₂O

2.1.1 6-Dimethylaminopurine (6-DMAP) (Sigma)

50mM, in H₂O

2.1.2 Barth

15mM Tris, 88 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 0.5 mM CaCl₂, pH 7.4

2.1.3 Bortezomib (Boston Biochem)

200 mM in Dimethyl Sulfoxide (DMSO)

2.1.4 Calcium Ionophore A23187 (Sigma)

10 mg/ml, in DMSO

2.1.5 Coomassie Stain Solution

40% (v:v) Ethanol, 10% (v:v) Acetic Acid, 0.1% (w:v) Coomassie Brilliant Blue R250 (VWR)

2.1.6 Coomassie Destain Solution

40% (v:v) Ethanol, 10% (v:v) Acetic Acid

2.1.7 Cycloheximide (CHX)

10 mg/ml, in H₂O

2.1.8 Cytochalasin D

10 mg/ml, in DMSO

2.1.9 D-Box Peptide

26 mM, in LFB1/50. D-Box peptide (RRTALGDVTNKVSE) (Peter et al., 2001) was custom synthesised by Insight Biotechnology

2.1.10 Dejelly Solution

2% (w:v) Cysteine in H₂O

2.1.11 Dynabead [150/500] Wash Buffer (DB150/500)

20mM Na₂HPO₄/NaH₂PO₄, 0.1% (v:v) Tween 20, either 150 or 500 mM NaCl, pH8.0

2.1.12 Energy Regenerator (ER)

10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 M Phosphocreatine Disodium Salt, 600 µg/ml Creatine Phosphokinase, pH 7.6

2.1.13 Extract Lysis Buffer (ELB)

10 mM HEPES, 50 mM KCl, 2.5 mM MgCl₂, 1 mM Dithiothreitol(DTT), 0.25M pH 7.7

2.1.14 Extract Dilution Buffer with Sucrose (EDB-S)

50 mM HEPES, 50 mM KCl, 10% (w:v) sucrose, 2 mM DTT, 0.4 mM MgCl₂, 0.4 mM Ethylene Glycol Tetraacetic Acid (EGTA), 1 µg/ml each of aprotinin, leupeptin and pepstatin, pH 7.6

2.1.15 Hoechst 33258

10 mg/ml, in H₂O (used at 20 µg/ml)

2.1.16 Licensing Factor Buffer 1 (LFB1)

40 mM HEPES, 20 mM K₂HPO₄/KH₂PO₄, 10% (w:v) sucrose, 50 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 2 mM DTT, pH 8.0. The number following LFB1 denotes the concentration of KCl, e.g. LFB1/50 contains 50 mM KCl

2.1.17 Licensing Factor Buffer 2 (LFB2)

LFB1 supplemented with 2.5 mM Mg-Adenosine Triphosphate (Mg-ATP)

2.1.18 Lysolecithin

5 mg/ml, in H₂O

2.1.19 Modified Marc's Ringer (MMR)

5 mM HEPES, 100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 0.1 mM Ethylenediaminetetraacetic acid (EDTA), pH 7.8

2.1.20 Nuclear Isolation Buffer (NIB); with Sucrose (NIBS)

50 mM HEPES, 50 mM KCl, 5 mM MgCl₂, 2 mM DTT, 0.1% (v:v) Triton X-100, 25 mM β-glycerophosphate, 0.5 mM spermidine-3HCL, 0.15 mM spermine-4HCL, pH8.0; NIB supplemented with 15% (w:v) Sucrose

2.1.21 Phosphate Buffered Saline with Tween 20 (PBS/T)

1 X PBS (Fisher), 0.02% (v:v) Tween-20

2.1.22 Protease Inhibitors

Aprotinin, 10 mg/ml, in H₂O

Leupeptin, 10 mg/ml, H₂O

Pepstatin, 10 mg/ml in Dimethylformamide

2.1.23 Proteinase K

20 mg/ml proteinase K, 10 mM Tris, 50% (v:v) glycerol, pH 7.5

2.1.24 Rehydration Buffer

30mM Tris, 7 M Urea, 2 M Thiourea, 1.2% (w:v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-Propanesulfonate (CHAPS), 0.4% (w:v) Amidosulfobetaine-14 (ABS-14), 0.25% (v:v) Ampholytes (GE Healthcare), 43mM DTT, pH 8.0

2.1.25 6 X Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) loading Buffer

440 mM Tris, 12% (w:v) SDS, 30% (v:v) Glycerol, 30% (v:v) β -Mercaptoethanol, 0.1% (w:v) Bromophenol Blue, pH 6.8

2.1.26 2Dimension (2D) SDS Buffer

50mM Tris, 25 mM DTT, 0.0002% (w:v) Bromophenol Blue, 6 M Urea, 2% (w:v) SDS, 30% (v:v) Glycerol, pH 8.8

2.1.27 Sepharose Wash Buffer

40 mM HEPES, 20 mM K_2HPO_4/KH_2PO_4 , 100 or 500 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 2mM EGTA, 10 % (w:v) sucrose, 10 μ g/ml each of aprotinin, leupeptin and pepstatin, pH 8.0

2.1.28 Stop C

20mM Tris, 5 mM EDTA, 0.5% (w:v) SDS, pH 7.5

2.1.29 SuNaSp

15 mM HEPES, 0.25 M sucrose, 75 mM NaCl, 0.5 M spermidine, 0.15 mM spermine, pH 7.6

2.1.30 Transfer Buffer

12.5 mM Tris, 85.9 mM Glycine, 10% (v:v) Methanol

2.1.31 Ubiquitin Reagents

His₆-Ubiquitin (His-Ub), Methylated Ubiquitin (M-Ub), UbiquitinK11R (K11R Ub), K11-only Ubiquitin (K11O Ub), UbiquitinK48R (K48R Ub), K48-only Ubiquitin (K48O Ub) were made up to 5 mg/ml in LFB1/50. Ubiquitin Vinyl Sulfone was supplied at 250 μ M in MES. All reagents were purchased from Boston Biochem.

2.1.32 XBE2

10 mM HEPES, 100 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 1.71% (w:v) sucrose, 5 mM EGTA, pH 7.7

2.2 Extract Preparation and Use

2.2.1 Frog Injections and Egg Collection

10-12 female frogs were treated with 50 units of Pregnant Mare Serum Gonadotrophin by subcutaneous injection three days before eggs were to be collected. One day before egg collection, frogs were treated with 500 units Chorionic Gonadotrophin (Chorulon) by subcutaneous injection to induce egg laying. Frogs were placed overnight into separate laying tanks containing approximately 2.5 L MMR. During egg collection only good quality eggs were pooled and tanks containing dirty buffer, activated, apoptotic or stringy eggs were discarded. Individual bad eggs from the pool of useful eggs were removed during the extract preparation using a plastic transfer pipette.

2.2.2 Preparation of Metaphase arrested Extract

Eggs were washed with MMR at room temperature to remove non-egg debris. Excess MMR was removed and eggs were incubated in dejelly solution. Dejelly solution was removed and replaced several times and eggs were swirled gently to encourage dejellying. This process takes approximately 10 minutes. Dejelled eggs were washed with XBE2 followed by 100 ml XBE2 supplemented with 10 µg/ml each of Aprotinin, Leupeptin and Pepstatin. Eggs were transferred to 14 ml Polypropylene tubes (Grenier BioOne) each containing 1 ml of XBE2 supplemented with 10 µg/ml each of Aprotinin, Leupeptin, and Pepstatin and 100 µg/ml Cytochalasin D. Eggs were packed by centrifugation at 1,400 x g for 1 minute in a Beckman JS-13.1 rotor at 16°C. Any activated eggs resting on the surface were removed with a plastic transfer pipette.

Eggs were then fractionated by crush-spinning at 16,000 x g for 10 minutes in a Beckman JS-13.1 rotor at 16°C. The cytoplasmic fraction was collected by puncturing the tube with a 19 G needle. All steps after this point were carried out at 4°C. Extract was supplemented with a 1:80 dilution of ER, LFB1/50 to 15% (v:v) and 10 µg/ml each of Cytochalasin D, Aprotinin, Leupeptin, and Pepstatin. Extract was centrifuged at 84,000 x g for 20 minutes in a Beckman SW55Ti rotor at 4°C.

The thick lipid layer was removed from the top of each tube with a spatula and the golden cytoplasmic layer was carefully collected by pipetting. Extract was supplemented to 1% (v:v) glycerol before being frozen in 25 µl, 100 µl and 200 µl aliquots in liquid nitrogen. Aliquots were stored at -80°C.

2.2.2.1 Testing Metaphase Arrest

Extracts were tested for their ability to maintain metaphase arrest for up to 6 hours by observing nuclear formation, or the lack thereof, by UV and light microscopy.

2.2.3 Using Metaphase Arrest Extracts

Extract was thawed in a room-temperature water bath and supplemented 1:40 with ER and 1:40 with 10 mg/ml Cycloheximide. Extracts were then stimulated to release from metaphase arrest by the addition of 0.3 mM CaCl₂. Extract was typically incubated at 20°C for 15 minutes to ensure complete release, unless otherwise stated.

2.2.4 Preparation of Nucleoplasmic Extract

Collected eggs were washed with MMR to remove non-egg debris. Dejelly solution was added and eggs were gently swirled at intervals. Eggs were washed three times with Barth and resuspended to a volume of 100 ml. Eggs were activated *in vivo* by the addition of 10 µl of 10 mg/ml Calcium Ionophore A23187 for 10 minutes at room temperature. This was carried out to avoid potential inconsistencies in extract quality that are associated with crush spin activation. Eggs were observed to have activated

after the vast majority rolled animal side up with contracting dark pigment. Eggs were washed three times with Barth, three times with cold ELB and were transferred to 14 ml Polypropylene tubes (Grenier BioOne). Eggs were centrifuged at 1,400 x g for 1 minute in a Beckman JS-13.1 rotor at 4°C. The supernatant and any apoptotic eggs were removed and eggs were centrifuged at 16,000 x g for 10 minutes in a Beckman JS-13.1 rotor at 4°C. The cytoplasmic fraction was gathered by puncturing the tube with a 19 G needle and placed in a 50 ml falcon tube on ice. This crude cytoplasm was used to generate nucleoplasmic extract (NPE)(Lebofsky et al., 2009), as outlined below.

Crude cytoplasm was supplemented 1:200 with 10 mg/ml Cycloheximide, 1:1000 with 10 mg/ml Aprotinin, 10 mg/ml Leupeptin and 1 M DTT, 1:2000 with 10 mg/ml Cytochalasin D (This deviates from the published protocol which used Cytochalasin B) and 1:1500 with 5 mg/ml Nocodazole and mixed gently by inversion, ensuring no bubbles were formed. Extracts were centrifuged at 20,000 x g in a Beckman JS-13.1 rotor for 20 minutes at 4°C. The top layer of lipids was completely removed with the bottom of a plastic pipette and a vacuum aspirator fitted with a gel loading tip. Extract was added to a 50ml tube on ice with care taken to avoid the dark granular material of the pellet. Extract was supplemented 1:40 with ER and 1:100 with 0.2 M ATP and mixed by inverting. Extract was transferred to 5 ml tubes (BD Falcon 352063) and warmed to room temperature for 5 minutes. 1 ml per tube was taken and 90 µl of 600 ng/ml sperm DNA was added and mixed thoroughly with a P1000 pipette 15 times before being returned to the 5ml tube. Extract and sperm reactions were mixed by inversion 10 times and incubated at 22°C. Further mixing was carried out by inversion, 5 times every 10minutes. At 60 minutes, assembly was monitored by adding 1 µl of the reaction mixture to 1 µl Hoechst 33258 and observing nuclear morphology by UV and light microscopy.

Once nuclei grew to an average diameter of 25-30 μm , which took 75-90 minutes, reactions were transferred to a glass round bottom tube (VWR 212-0030) inside a 14 ml Falcon containing 3ml cold MilliQ H_2O on ice. Water levels were adjusted so that the meniscus was at the same height as the meniscus of the extract. Reactions were centrifuged at 19,000 x g in a Beckman JS-13.1 for 3 minutes at 4°C. Nuclei were removed from the top with a cut-off P200 pipette tip and added to a 1.5 ml tube on ice. Nuclei were transferred to 5 x 20 mm tubes (Beckman 342630) using a cut off P200 pipette tip and centrifuged at 259,000 x g in a Beckman TLS-55 with 358614 adaptors for 30 minutes at 2°C. Tubes were placed on ice and any lipids were removed with a gel loading tip equipped vacuum pump. NPE was transferred to a new tube, avoiding contamination from membranes and chromatin pellets, and frozen in 20 μl aliquots on liquid nitrogen.

2.2.5 Preparation of Pre-Incubated Extract

Metaphase extract released for 10 minutes with 0.3 mM CaCl_2 was supplemented with sperm DNA to a final concentration of 10 ng/ μl and incubated at 20°C for 90 minutes. Extracts were diluted with 3 volumes of ice cold LFB2/50 and mixed vigorously before being centrifuged in 7 x 21 mm polycarbonate thickwall tubes (Beckman 343775) at 230,000 x g in a Beckman TLA-100 for 20 minutes at 4°C. The supernatant was recovered and frozen in liquid nitrogen in 25 μl aliquots.

2.2.6 Sperm Nuclei Preparation

15 male frogs were primed with 50 units of Chorionic Gonadotrophin (Chorulon) 5-9 days prior to removal of the testes. Frogs were euthanised in MS222 (0.2% (w:v) Tricaine mesylate MS222, 0.5% (w:v) NaHCO_3 , pH 7.5). Testes were removed and washed in EB. Extraneous tissue and blood vessels were removed with dissection forceps. Testes were transferred to a petri dish containing 10 ml EB and were finely

chopped with a razor blade. Chopped material was pooled and filtered through a 25 μm nylon filter and spun at 2,000 x g in a swinging-bucket rotor for 5 minutes at 4°C, this spin was repeated until the supernatant no longer appeared cloudy. The pellet was resuspended in a total volume of 0.5 ml SuNaSp per testis. This was supplemented with 25 μl of 5 mg/ml Lysolecithin per testes and incubated at room temperature for 5 minutes. 1 μl sperm was mixed with 1 μl of 20 $\mu\text{g/ml}$ Hoechst 33258 and examined for demembration by UV microscopy. Demembration of sperm by Lysolecithin allows staining to occur. If <95% of sperm appeared stained under UV then Lysolecithin treatment was repeated. Demembrated sperm were then centrifuged at 2,000 x g in a swinging-bucket rotor for 5 minutes at 4°C. Lysolecithin was quenched by resuspending pellets in 0.5 ml SuNaSp containing 3% (w:v) BSA for each testis. Sperm were centrifuged again and the pellet washed by resuspension in 0.5 ml EB for each testis. The wash step was repeated and sperm were resuspended in 100 μl EB containing 30% (v:v) glycerol for each testis. DNA content was calculated, assuming a *Xenopus* haploid genome contains 3 pg of DNA, by counting the number of sperm and somatic cells in a 1:100 dilution of sperm preparation in EB on a haemocytometer. Stock was diluted in EB 30% (v:v) glycerol to give a final concentration of 400 ng/ μl DNA and aliquots were stored long term at -80°C. Working aliquots of DNA were kept at -20°C.

2.3 Assays

2.3.1 DNA Replication assays and TCA precipitation

Extracts were supplemented with 0.3 μCi α -³²P dATP, released by the addition of 0.3 mM CaCl_2 and incubated for 15 minutes at 20°C. DNA was added at 6 ng/ μl , unless otherwise stated, extract was mixed thoroughly and aliquoted into 10 μl per timepoint. Reactions, which typically lasted 90 minutes, were terminated by the addition of 160 μl Stop C containing 0.2 mg/ml Proteinase K and incubated at 37°C for 30 minutes. DNA was precipitated by adding samples to snap-cap tubes (BD Falcon 352063) containing 4

ml ice-cold 10% TCA, 2% (w:v) $\text{Na}_4\text{P}_2\text{O}_7$ and incubating at 4 °C for 30 minutes. Precipitated material was resuspended by inverting the tubes. 40 μl of TCA samples were spotted onto paper filters for the measurement of 1% total counts of ^{32}P . The remainder of samples were filtered under vacuum through glass microfibre filters. Empty tubes were rinsed with 10% TCA and this was applied to the vacuum filter. Filters were washed with 5% TCA, 0.22% (w:v) $\text{Na}_4\text{P}_2\text{O}_7$ followed by 100% (v:v) ethanol. Paper and glass filters were dried under infra-red light. ^{32}P on the dried filters was measured by scintillation counting in 1 ml Optiscint. The percentage of total counts incorporated (%TC) was calculated as follows:

$$\%TC = \text{Sample counts} / (1\% \text{ Total counts} \times 99)$$

Assuming an endogenous concentration of 50 μM dATP in extract (Blow and Laskey, 1986), the total dATP incorporation is calculated as follows:

$$\begin{aligned} [\text{dATP incorp.}] &= (\%TC / 100) \times 50 \mu\text{M} \\ &= \%TC / 2 \mu\text{M} \end{aligned}$$

Assuming the ratio of GC:TA is 1:1, then total dNTP incorporation can be calculated as follows:

$$\begin{aligned} [\text{dNTP incorp.}] &= (\%TC / 2 \mu\text{M}) \times 4 \\ &= \%TC \times 2 \mu\text{M} \\ &= \%TC \times 2 \mu\text{moles/litre} \end{aligned}$$

Assuming an average molecular weight for dNMP of 327, then dNMP incorporation can be calculated as follows:

$$\begin{aligned} [\text{dNMP}] &= \%TC \times 2 \times 327 \mu\text{g/litre} \\ &= \%TC \times 0.654 \text{ ng}/\mu\text{l}. \end{aligned}$$

2.3.2 D-box Assay

Extract supplemented with 0.3 μCi $\alpha\text{-}^{32}\text{P}$ dATP was activated by addition of 0.3 mM CaCl_2 . At 2, 4, 6, 8, 10, 12 and 14 minutes after activation 10 μl aliquots were added to

tubes containing 0.76 μ l of 26 mM D-box peptide (2 mM final concentration) and 0.5 μ l of 100 ng/ μ l sperm DNA (5ng/ μ l final concentration) and mixed carefully by pipetting. Samples were incubated with DNA for 3 minutes to allow for minimal licensing and subsequently supplemented with 0.5 μ l of 2 μ M geminin^{DEL} (100 nM final concentration) to prevent further licensing. Reactions were incubated for 90 minutes before DNA replication was measured by TCA precipitation.

2.4 Chromatin Isolation

Extracts supplemented with sperm (final concentration of 10 ng/ μ l) were aliquoted into 10 μ l samples per timepoint. Samples were diluted 1:50 with NIB and 100 μ l NIBS (NIB supplemented with 15 % (w:v) sucrose) was carefully added to the bottom of the tube. Chromatin was pelleted by centrifugation at 1,800 x g in a swing bucket rotor at 4°C. NIB was removed and the sucrose pellet was washed once with 100 μ l NIB. The NIBS cushion was removed to leave 15 μ l remaining per tube. This was centrifuged at 23,000 x g for 2 minutes in a fixed angle rotor at 4°C to concentrate the chromatin pellet at the tube wall. Supernatant was removed and the chromatin was resuspended in SDS-PAGE loading buffer. Samples were analysed by SDS-PAGE. Histones were analysed by Coomassie staining as a loading control and proteins of interest analysed by western blotting.

2.5 Antibody Techniques and Protein Pulldowns

2.5.1 Antibody Bead Preparation for Immunodepletion

Protein A Sepharose beads were washed four times with 100 mM Hepes, pH 8.0. Beads were recovered by centrifugation in a swing bucket rotor at 2,000 x g for 2 minutes at 4°C. 2 volumes of antibody serum, adjusted to 100 mM Hepes, pH 8.0 was added to the beads and tubes were placed securely inside a 50 ml falcon tube for a 1 hour incubation on a roller at room temperature. Beads were washed three times with 100 mM Hepes,

pH 8.0 and once with 100 mM Hepes, pH 8.0 freshly supplemented with 1 mM Phenylmethylsulfonyl Fluoride (PMSF). Beads were washed three times in 100 mM Hepes, pH 8.0 to remove the PMSF followed by three washes in LFB1/50. All wash steps were ten times the bed volume.

2.5.2 Immunodepletion

Buffer was completely removed from pre-prepared Protein A-antibody beads with a gel loading tip. Beads were added to 200 µl of extract and mixed by gently tapping the tube. For Cdt1 depletion, beads were added at 70% of extract volume. For geminin depletion beads were added at 40% of extract volume. Tubes were placed securely inside a 50 ml falcon tube and samples were mixed on a roller for 40 minutes at 4°C. Extracts were spun through a 25 µm Nybolt filter to remove beads and for both Cdt1 and geminin a second round of depletion was carried out before being frozen in liquid nitrogen and stored at -80°C.

2.5.3 Antibody Affinity purification

All solutions were filtered through a 0.2 µm filter and were applied to the column using a peristaltic pump (Watson Marlow 505S) at a flow rate of 1 ml/minute. A 1 ml HiTrap NHS-activated HP column (GE Healthcare) was activated with 6 ml of ice-cold 1 mM HCl. 1 ml of antigen above a concentration of at least 0.5 mg/ml in coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3) was added to the column and the column was let stand for 30 minutes. Alternatively, larger volumes of antigen at concentrations below 0.5 mg/ml were circulated through the column for 30 minutes. Columns were washed with 10 ml of Blocking Buffer (0.5 M Ethanolamine, 0.5 M NaCl, pH 8.3) and left to stand for 30 minutes to ensure all unreacted NHS-groups were blocked. Columns were washed sequentially with 10 ml of 10 mM Tris, pH 8.0, 10ml of 0.1 M glycine, pH

2.0, 10 ml of 10 mM Tris, pH 8.0 and 10 ml of 0.1 M Triethylamine, pH 11.5. All washes were repeated before washing with 30 ml of PBS.

15 ml of serum was diluted 1:2 with 2 X PBS and NaN_3 was added to a final concentration of 0.1% (v:v). Serum was filtered through a 0.2 μm filter before being applied to the column, recirculating overnight at 4°C. The column was washed with 50 ml of Washing Buffer (1 X PBS, 0.5 M NaCl, 0.1% (v:v) Triton X-100) followed by 50 ml of 1 X PBS. Antibodies were eluted from the column with 0.1 M glycine, pH 2.6. 1 ml of elute was collected in 1.5 ml Eppendorf tubes containing 100 μl of 2 M Tris, pH 8.5. Antibodies were quantified on a Nanodrop 2000 (Thermo) and the highly concentrated fractions were pooled and dialysed against 1 X PBS. Glycerol was added to a final concentration of 30% (v:v) and antibodies were stored at -80°C. Some peptides used for affinity purification were insoluble and purified under denaturing conditions. To prevent precipitation these peptides were dialysed into coupling buffer containing 1 M urea.

2.5.4 Crosslinking of Protein A/G Dynabeads

50 μl of beads were placed in a magnet (DynaL MPC-P-12), supernatant was removed and beads were resuspended in 200 μl of PBS/T. 1.66 μl of antibody serum or 20 μg of affinity purified IgG was added and incubated with rotation at room temperature for 15 minutes. Beads were washed twice with 200 μl of PBS/T and twice with 200 μl Conjugation Buffer (20 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 0.15 M NaCl, pH 7.5). 200 μl of 5 mM Bis[sulfosuccinimidyl] suberate (Thermo) (made up in conjugation buffer) was added and antibody bound beads were incubated for 30 minutes at room temperature with rotation. Reactions were stopped by addition of 12.5 μl 1 M Tris, pH 7.5 for 15 minutes at room temperature with rotation. Beads were washed three times with 200 μl PBS/T and stored at 4°C for up to 2 months.

2.5.6 Immunoprecipitation

50 µl of metaphase arrested or interphase extract was diluted 1:5 with ice-cold LFB1/50. Extracts were centrifuged, to remove any precipitates or aggregates, at 14,000 rpm for 20 minutes at 4°C in a fixed angle rotor. 1-5 % (v:v) serum or 1-20 µg purified antibody was added and mixed well. The extract/antibody mixture was incubated for 1 hour on ice or at 4°C. 10 µl of pre-washed Protein A/G Sepharose or 20 µl Protein A/G Dynabeads was added to the extract/antibody mixture, mixed well and rotated at 4°C for 1 hour. Protein A/G agarose beads were washed three times with 400 µl of Sepharose wash buffer containing 100 mM, 500 mM and 100 mM respectively. Protein A/G Sepharose was recovered, and unbound proteins in the wash supernatant separated out, by centrifugation at 2000 x g for 2 minutes at 4°C in a swinging bucket rotor. Protein A/G Dynabeads were recovered in a magnetic stand on ice. Beads were washed three times with 200 µl of Dynabead 150, Dynabead 500 and Dynabead 150. Proteins were recovered from the beads by boiling in SDS-PAGE loading buffer for 5 minutes at 95°C.

2.5.7 Geminin 10 Minute Immunoprecipitation

Metaphase or interphase extract was diluted 1:2 with ice-cold LFB1/50 and mixed 1:1 with Protein A Dynabeads crosslinked to geminin antibody serum. Samples were mixed by rotation for 10 minutes at 4°C before being placed in a magnet for 1 minute. 200 µl of DB150 was added and mixed thoroughly to reduce viscosity and allow beads to pellet. Beads were washed a further 2 times, with 200 µl of DB500 followed by DB150. Beads were boiled for 5 minutes in SDS PAGE loading dye.

2.6 SDS-PAGE and Western blotting

2.6.1 SDS-PAGE

Samples were boiled for 5 minutes in SDS-PAGE loading dye. Samples were spun in a benchtop centrifuge to incorporate any condensation and vortexed briefly. Samples were loaded onto 12 or 20 well 4-12% Bis-Tris NuPAGE gels (Life Technologies) (unless stated otherwise). Electrophoresis was carried out at 80 V for an initial 15 minutes to allow for efficient stacking prior to being run at 170 V in 1X NuPAGE MOPS Running Buffer (Life Technology).

2.6.2 Coomassie Staining

SDS-PAGE Gels were incubated in Coomassie stain overnight. Destain was added and refreshed at intervals of 45 minutes until sufficiently destained. Gels were incubated in water to reswell before scanning on an EPSON Perfection V500 scanner at 800 dpi.

2.6.3 Western Blotting

Gels were transferred onto Hybond Polyvinylidene Fluoride (PVDF) (GE Healthcare) in Transfer Buffer for 2 hours at 80 V using a Biorad 2.5L Trans Blot cell on ice. Membranes were blocked in 5 % (w:v) skimmed milk (Marvel) in PBS/T for 1 hour at room temperature and washed in PBS/T for 10 minutes. Primary antibodies, typically in 3% (w:v) BSA PBS/T, were incubated for 1-2 hours at room temperature and blots were washed in PBS/T for 10 minutes. HRP-conjugated secondary antibody was incubated for one hour at room temperature and blots were washed for 20 minutes. Blots were incubated for 5 minutes in Supersignal West Pico ECL (Thermo) before being exposed on film (Kodak) or in a Fujifilm LAS4000 camera. Film was scanned on an EPSON Perfection V500 at 800 dpi and images were prepared in Photoshop. Fujifilm images were saved as TIFF files, processed in Photoshop and only images acquired with the

camera were used for the quantification of bands, carried out with Aida imaging software.

Protein Target	Species	Manufacturer	Dilution
TopBP1	Sheep	A. Gambus/Blow Lab	1:1000
Mcm2	Rabbit	(Thömmes et al., 1997)	1:3000
Mcm3	Rabbit	(Mahbubani et al., 1997)	1:3000
Mcm7	Mouse	(Thömmes et al., 1997)	1:3000
Cdt1	Rabbit	(Tada et al., 2001)	1:1000
Cdc45	Sheep	(Gambus et al., 2011)	1:2000
Cyclin B2	Rabbit	J. Gannon	1:2000
Geminin	Rabbit	(Tada et al., 2001)	1:2000
GemC1(1-97)	Rabbit	K. Creavin/Blow Lab	1:1000
His₆	Mouse	GE Healthcare	1:1000
Anti-Mouse HRP	Donkey	GE Healthcare	1:10000
Anti-Rabbit HRP	Donkey	Cell Signalling	1:5000
Anti-Sheep HRP	Donkey	Sigma	1:2500

Table 1: Antibody Conditions for Western Blotting

2.7 2D Gel electrophoresis

Whole extract or immunoprecipitated geminin samples were denatured in Rehydration buffer for 2 hours at room temperature with shaking. Samples were supplemented 1:150 with 9M acrylamide and were alkylated for 1.5 hours at room temperature with shaking. Samples were centrifuged at 16,000 x g for 10 minutes in a fixed angle rotor to remove any particulate matter. pH 3-5.6 NL 7 cm or pH 3-10 NL 24 cm Immobilon DryStrips (GE Healthcare) were rehydrated overnight. For 7 cm strips the max volume was 125 µl and max protein load was ~50 µg. Samples were prepared with 0.5 µl of whole extract or with a geminin immunoprecipitate from 200 µl of extract. For 24cm strips the max volume was 400 µl and max protein load was ~500 µg. Samples were prepared with 10 µl of whole extract or with a geminin immunoprecipitate from 800 µl of extract. Isoelectric focusing was carried out using an Agilent 3100 OFFGEL fractionator. A focusing charge was applied at 250 V for 30 minutes before a ramp to 5,500 V, which was applied for a minimum of 33,000 Voltage Hours.

Strips were incubated in 2D SDS buffer for 30 minutes at room temperature before proteins were separated by SDS-PAGE on 4-12% Bis-Tris ZOOM gels. Gels were then subjected to western blotting or alternatively protein staining and gel excision of spots for mass spectrometry.

2.8 Analytical Size exclusion chromatography

Analytical gel filtration experiments were performed on a Thermo Ultimate 3000 HPLC system.

2.8.1 MAbPAC SEC-1 (Thermo)

Samples were centrifuged through a 0.2 µm PVDF filter (Millipore) to remove particulate matter prior to injection. Typically 38 µl samples, equivalent to 1% column volume, were injected on a 4 x 300 mm 5 µm MAbPAC SEC-1 column equilibrated with ice-cold LFB1/200 (NaCl instead of KCl) at 150 µl/min. A total of 24 x 75 µl (30 seconds) fractions were collected from 1.2 - 3 ml (8 - 20 minutes). Fractions were analysed by SDS-PAGE followed by Coomassie Staining or Western blotting.

2.8.2 Superose 6 (GE Healthcare)

Samples were centrifuged through a 0.2 µm PVDF filter (Millipore) to remove particulate matter prior to injection. 20 µl samples were injected on a 2.4 ml Superose6 PC 3.2/30 equilibrated with ice-cold buffer (Specific Buffers mentioned in the text) at 25 µl/min. A total of 24 x 50 µl (120 seconds) fractions were collected from 0.8 - 2 ml (32 - 80 minutes). Fractions were analysed by SDS-PAGE followed by Coomassie Staining or Western blotting.

2.9 Size Exclusion Chromatography coupled to Multi Angle Light Scattering

Size Exclusion Chromatography coupled to Multi Angle Light Scattering (SEC-MALS) experiments were performed on a Thermo Ultimate 3000 HPLC system with an inline miniDAWN TREOS multiangle light scattering detector (Wyatt) and Optilab T-rEX differential refractive index (dRI) detector (Wyatt). Prior to performing SEC-MALS experiments a Superdex200 10/300 GL (GE Healthcare) or MAbPac SEC-1 (Thermo) column was equilibrated overnight at 300 μ l/min or 150 μ l/min, respectively, with 0.1 μ m filtered buffer (40 mM HEPES, pH 8.0, 200 or 1000 mM KCl) and the differential refractive index (dRI) detector's reference cell was purged with the running buffer. For accurate mass determination 150 μ l (for Superdex) or 40 μ l (for MAbPac) of recombinant geminin at 2 mg/ml was injected onto the column when flat baselines were observed for all light scattering (LS) angle detectors and the dRI index detector with background scattering in LS detector 2 not in excess of 0.0001 Volts. Molar masses across elution peaks were calculated using ASTRA v6.0.0.108 software (Wyatt).

2.10 Glycerol Gradients

100 μ l of extracts diluted 1:5 with LFB1/50 or molecular weight markers were layered carefully on 4ml linear 5-50% (v:v) or 5-28% (v:v) glycerol gradients in LFB1/200 and centrifuged in a Beckman SW60Ti rotor at 363,137 x g for 16 hours at 4 °C. 200 μ l samples were collected.

2.11 Mass Spectrometry

2.11.1 Sample preparation

All sample preparation was performed with the help of GRE proteomics. Samples were resolved by SDS-PAGE, gels were stained with Instant blue stain (Expedeon) and gel

pieces were excised with a clean scalpel. Gel pieces were washed twice with 150 μ l 50 mM NH_4HCO_3 , 100 % (v:v) Acetonitrile (ACN) for 10 minutes at room temperature with shaking. Supernatant was discarded and 100 μ l of 100 % (v:v) ACN was added prior to addition of 100 μ l of NH_4HCO_3 . Gel pieces were incubated at 37 °C with shaking for 30 minutes. Supernatant was removed and gel pieces were dried completely in a vacuum centrifuge at 45°C. Proteins were reduced with 50 μ l 10 mM DTT for 45 minutes at 55°C with shaking. DTT was removed and proteins were alkylated with 50 μ l of 55 mM Iodoacetamide in the dark at room temperature for 30 minutes. Supernatant was removed and gel pieces were washed twice with 150 μ l of 50 mM NH_4HCO_3 , 100 % (v:v) ACN for 10 minutes with shaking. Gel pieces were dried completely in a vacuum centrifuge at 45°C. 50 μ l of Trypsin (20 μ g/ml in 50 mM NH_4HCO_3 pH 8.0(Thermo)) or Lys-C(20 μ g/ml in 50 mM NH_4HCO_3 pH 8.0 (Thermo)) was added and gel pieces were incubated overnight at 37°C. 50 μ l of 100% (v:v) ACN was added and samples were sonicated in an ice cold sonication bath for 15 minutes to release peptides. Supernatant was collected. Sonication of gel pieces was repeated with 100 μ l 70% (v:v) ACN, 0.1%(v:v)Trifluoroacetic acid (TFA) and the supernatants were combined. Sample volumes were reduced to ~60 μ l in a vacuum centrifuge at 60°C. C18 ‘Ziptips’ were prepared by inserting a small circle of C18 filter disk as a frit into a gel loading tip. Tips were then loaded with 10 μ l of 0.1% (v:v) TFA and 7 μ l of a 1:1.4 slurry of POROS C18 in 70% (v:v) ACN, 0.1% (v:v) TFA. C18 ‘Ziptip’s’ were activated with 20 μ l 50% (v:v) ACN, 0.1%(v:v) TFA and washed with 20 μ l 0.1% (v:v) TFA acid before loading 60 μ l of sample peptides. Samples were washed with 20 μ l 0.1% (v:v) TFA and 40 μ l 50% (v:v) ACN, 0.1%(v:v) TFA was added twice to elute bound peptides. Sample volumes were reduced to ~10 μ l in a 60°C vacuum centrifuge.

2.11.2 Sample Submission

Samples were submitted to the Fingerprints Proteomics Facility, University of Dundee by GRE proteomics. 10 µl samples were analysed on a LTQ Orbitrap Velos Pro (Thermo).

2.11.3 Data Analysis

Raw Spectral data were analysed using MaxQuant version 1.0.13.13. The fixed modification selected was Carbamidomethylation (C) and specific variable modifications, such as Acetylation (K) and Methylation (K/R) were selected.

2.12 Recombinant Protein: Cloning and Expression

2.12.1 Cloning: His6-Geminin

A full length *Xenopus laevis* Geminin H pET28(a) plasmid, which adds an N-terminal His₆-tag, was kindly gifted by Dr. Thomas J. McGarry (McGarry and Kirschner, 1998). This plasmid was found to have an N-terminal insertion of 81 amino acids between the His₆-tag and the protein start methionine (Figure 49). PCR primers with flanking restriction sites were designed with homology to the geminin start methionine and stop codon. Primers were custom synthesised by Oligonucleotide Synthesis Service, University of Dundee.

Forward primer with NdeI insertion (5' - 3'):

CCC CCC CAT ATG AAT ACC AAC AAG AAG CAG AGA TTG

Reverse primer with BamHI insertion (5' - 3'):

CCC CCC GGA TCC CTA GAC AGT ATG TGC ATC CAT ATT C

Full length wild type geminin was PCR amplified from the original plasmid using the following recipe and the cycle was repeated 30 times using an Eppendorf Mastercycler Thermal Cycler:

	Concentration	Volume (μl)	Temperature (°C)	Time (sec)
Plasmid	100 ng/μl	5	98	18
Forward Primer	10 μM	2.5	98	30
Reverse Primer	10 μM	2.5	65	30
Phusion Mix	2 X	25	72	30
Water		15	72	120
Total		50	22	Hold

Table 2: PCR Recipe and Cycle Conditions

PCR products were run on 0.8% (w:v) agarose gel and DNA was visualised using Ethidium bromide. The band corresponding to the correct size was excised from the gel and prepared using a QIAquick gel extraction kit (Qiagen). Both the PCR amplified insert as well as the target vector, pET15b (Novagen), were digested with NdeI and BamHI (New England BioLabs) in order to generate complementary sticky DNA overhangs for ligation. Restriction digestions were performed at 37°C in Cutsmart buffer (New England BioLabs) for 1 hour. For the pET15b plasmid a sequential double digest was carried out followed by Calf intestinal alkaline phosphatase treatment (New England Biolabs) at a final concentration of 10U/reaction for 1 hour at 37°C. Restriction digested DNA and PCR products were run on 0.8% (w:v) agarose gel, bands were excised and DNA prepared using a QIAquick gel extraction kit followed by a final cleanup with GeneJET PCR Purification kit (Thermo). Insert and target vector were ligated using T4 DNA ligase (New England BioLabs) using a 3-fold molar excess of insert. Ligation reactions were performed at room temperature for 1 hour. 5 μL of ligated DNA product was then transformed into competent cells. Single bacterial colonies were picked from Lysogeny broth (LB) agar selection plates and used to inoculate pre-warmed LB media supplemented with Ampicillin. Clones were incubated overnight at 37°C with shaking. Preparation of plasmid DNA was carried out using a

QIAprep kit (Qiagen). Sequence validation was carried out by DNA sequencing performed in-house using the Dundee sequencing service.

2.12.2 Recombinant Protein Expression: His₆-Tagged Geminin

Lemo21(DE3) Competent *E. coli* (New England BioLabs) were transformed to express His₆-tagged wildtype *Xenopus laevis* geminin. Glycerol stocks were made of a highly expressing clone. A P200 pipette tip was used to scrape the glycerol stock and inoculate a 100 ml starter culture of LB supplemented with Ampicillin and Chloramphenicol. Starter culture was either incubated at 37°C for 5-6 hours or at room temperature overnight. 7 ml of confluent starter culture was used to inoculate 500 ml cultures which were pre-warmed to 37°C. At an OD₆₀₀ of 0.5-0.7 the temperature was reduced to 25°C and cultures were induced to express protein by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 3 hours cultures were combined and centrifuged in a Beckman JLA-8.1000 rotor at 15,900 x g for 10 minutes at 4°C.

Cells from 3 L of culture were resuspended in 30 ml of 40 mM HEPES, 100 mM KCl, pH 8.0 containing Complete EDTA Protease inhibitor cocktail (Roche). Cells were lysed partially by freeze-thawing. Cells were frozen in liquid nitrogen and thawed in a room temperature water bath. After cells had thawed for 10 minutes, Benzonase (25 Units/μl) was added at 1:1000 and lysates were returned to the waterbath. Once lysates were thawed completely (around 20 minutes), lysates were placed on wet ice and subjected to sonication for 60 seconds (10 seconds on, 20 seconds off) at 30% amplitude. Lysates were centrifuged at 10,000 x g in a Beckman JA-25.50 rotor for 20 minutes at 4°C, supernatants were collected and supplemented with Imidazole to a final concentration of 15 mM. Ni-NTA slurry (Life Technologies) was equilibrated with 40 mM HEPES, 100 mM KCl, 15 mM Imidazole, pH 8.0 and added to the lysate for batch

binding. Lysates were rolled for 1 hour at 4°C before being applied to a 10 ml disposable polypropylene column (Life Technologies).

The column was sequentially washed with 50 ml of 40 mM HEPES, 100 mM KCl, 30 mM Imidazole, pH 8.0, 50 ml of 40 mM HEPES, 500 mM KCl, 30 mM Imidazole, 0.1% (v:v) Triton X-100, pH 8.0 and 50 ml of 40 mM HEPES, 100 mM KCl, 30mM Imidazole, pH 8.0. Protein was eluted off the column with 15 ml of 40 mM HEPES, 100 mM KCl, 300 mM Imidazole, pH 8.0. Eluted protein was concentrated in a 30,000 kDa molecular weight cutoff centrifugal filter unit (Millipore) to 700 µl before being applied to a Superdex 200 10/300 GL. Proteins were fractionated at 300 µl/min in 40 mM HEPES, 100 mM KCl, pH 8.0 on a Thermo Ultmate3000. Geminin fractions were pooled and frozen in liquid nitrogen or concentrated to 2 mg/ml for SEC-MALS.

2.12.3 Recombinant Protein Expression: His₆-Tagged Cdt1

A full length *Xenopus laevis* Cdt1 prSETb plasmid, which adds an N-terminal His₆-tag, was kindly gifted by Dr. Marcel Méchali. BL21 (DE3) *E coli*(Maiorano et al., 2000). (Novagen) transformed with this plasmid were found to produce recombinant protein in the absence of IPTG induction, which likely had a toxic effect on cells. Cells lines such as Rosetta 2 (Novagen) which are designed to stop leaky expression were found to produce very little protein after induction with IPTG. BL21 (DE3) cells were used for expression but measures were taken to ensure that these cells never reached a stationary phase after being woken from glycerol stocks.

A P200 pipette tip was used to inoculate a 30 ml starter culture of LB supplemented with Ampicillin. Starter cultures were incubated at room temperature overnight for 10-12 hours. 5 ml of starter culture was used to inoculate 500 ml cultures which were pre-warmed to 37°C. At an OD₆₀₀ of 0.4-0.6 the cultures were induced to express protein by addition of 1 mM IPTG. After 3 hours cultures were combined and centrifuged in a

Beckman JLA-8.1000 rotor at 15,900 x g for 10 minutes at 4°C. Cells from 2 L of culture were lysed in 35 ml BugBuster (1 X BugBuster Extraction Reagent (Novagen), 40 mM HEPES, 50 mM KCl, pH 8.0) supplemented with Complete EDTA Protease inhibitor cocktail (Roche) and Benzonase (25 Units/μl) at 1:1000. Cells were lysed by pipetting and were placed on a roller at room temperature for 30 minutes. Lysates were centrifuged at 16,000 x g in a Beckman JA-25.50 rotor for 20 minutes at room temperature.

Cdt1 was expressed in an insoluble form and supernatants were discarded. Pellets containing Cdt1 were resuspended with 20 ml of Solubilisation Buffer (40 mM HEPES, 20 mM K₂HPO₄/KH₂PO₄, 200 mM KCl, 8 M Urea, pH 8.0) and were left on a roller overnight at room temperature. Insoluble material was removed by centrifugation at 16,000 x g in a Beckman JA-25.50 rotor for 20 minutes at room temperature. The supernatant was supplemented with 15 mM Imidazole and added to 2 ml of pre-equilibrated Ni-NTA slurry. Batch binding was carried out on a roller for 3 hours at room temperature. The beads mixture was applied to a 10 ml disposable polypropylene column (Life Technologies) and the flowthrough was discarded. The column was washed twice with 25 ml of Cdt1 Wash Buffer (40 mM HEPES, 500 mM KCl, 8 M Urea, 30 mM Imidazole, 0.03% (v:v) Triton X-100, pH 8.0) and proteins were eluted with 10 ml of Cdt1 Elution Buffer (40 mM HEPES, 200 mM KCl, 8 M Urea, 300 mM Imidazole, 0.03% (v:v) Triton X-100, pH 8.0).

Purified insoluble Cdt1 was refolded in 3 ml Pur-A-Lyzer Maxi 12000 (Sigma) at 4°C by a stepwise dialysis in THED200 (0.03% Triton, 20mM HEPES, 20% (v:v) Ethylene Glycol, 1 mM DTT, 200 mM KCl) supplemented with various amounts of Urea beginning with 4 M overnight and followed sequentially by 2 M, 1 M, 0.5 M, 0 M and 0

M for 3 hours each. Purified refolded His₆-Cdt1 was frozen in 20 µl beads in liquid nitrogen and was stored in liquid nitrogen.

2.12.4 His₆-Tagged GemC1

A full length *Xenopus laevis* GemC1 p-DEST17 plasmid, which adds an N-terminal His₆ tag, was a kind gift from Dr. Vincenzo Costanzo (Balestrini et al., 2010). BL21 (DE3) *E. coli* (Novagen) cells were transformed and a highly expressing clone was picked to make a glycerol stock. Glycerol stocks were used to inoculate 30 ml of LB supplemented with Ampicillin and cultures were incubated at 37°C overnight with shaking. 5 ml of overnight culture was added to 500 ml LB supplemented with Ampicillin and incubated at 37°C. At an OD₆₀₀ of 0.6 - 0.8 cultures were induced with 1 mM IPTG. After 3 hours at 37°C cultures were combined and centrifuged in a Beckman JLA-8.1000 at 15,900 x g for 10 min at 4°C. Cells from 2 L of culture were lysed in 35 ml BugBuster (1 X BugBuster Extraction Reagent (Novagen), 40 mM HEPES, 50 mM KCl, pH 8.0) supplemented with Complete EDTA Protease inhibitor cocktail (Roche) and Benzonase (25 Units/µl) at 1:1000. Cells were lysed by pipetting and were placed on a roller at room temperature for 30 minutes. Lysates were centrifuged at 16,000 x g in a Beckman JA-25.50 rotor for 20 minutes at room temperature.

His₆-GemC1 was insoluble and was found in the pellet. Pellets were solubilised in 20 ml of IMAC5 (20 mM Tris, 0.5 M NaCl, 5 mM Imidazole, 8 M urea, pH 8.0) on a roller overnight. Insoluble material was removed by centrifugation at 16,000 x g in a Beckman JA-25.50 rotor for 20 minutes at room temperature. Supernatant was applied to pre-equilibrated 2 ml slurry of Ni-NTA in a 10 ml disposable column. Columns were washed 3 times with 10 ml of IMAC5 supplemented with 20 mM Imidazole. Proteins were eluted with 10 ml of IMAC5 supplemented with 300 mM Imidazole and collected in 1 ml fractions. Fractions were analysed by SDS-PAGE and Coomassie staining and

crudely quantified against BSA standards. Fractions were combined to give concentrations of ~0.3 mg/ml of GemC1, as higher concentrations were found to precipitate during dialysis. Proteins were refolded in a step-wise dialysis in 20mM HEPES, 200mM KCl, 1mM DTT, pH8.0 starting with 4M urea overnight at 4°C, followed by 2 hours each at 2 M, 1M, 0.5 M and then dialysed twice in dialysis buffer containing no urea.

2.12.5 MBP-Tagged GemC1

A full length *Xenopus laevis* GemC1 pMAL-c4X plasmid, which adds an N-terminal Maltose Binding Protein (MBP) tag, was a kind gift from Dr. Vincenzo Costanzo (Balestrini et al., 2010). BL21 (DE3) *E. coli* (Novagen) cells were transformed and a highly expressing clone was picked to make a glycerol stock.

Glycerol stocks were used to inoculate 30 ml of LB supplemented with Ampicillin and cultures were incubated at 37°C overnight with shaking. 5 ml of overnight culture was added to 500 ml LB supplemented with Ampicillin and incubated at 37°C. At an OD₆₀₀ of 0.4 the temperature was reduced to 18°C. Cultures were induced to express protein with 1 mM IPTG for 4 hours. Cultures were combined and centrifuged in a Beckman JLA-8.1000 at 15,900 x g for 10 min at 4°C. Cells from 2 L of culture were resuspended in 25 ml ice cold 1 X PBS supplemented with Complete EDTA Protease inhibitor cocktail (Roche). Lysozyme was added at 1 mg/ml and cultures were rotated at 4°C for 30 minutes. Triton X-100 was added to 1% (v:v) and cells were sonicated at 50% amplitude for 20 seconds on ice. Partially lysed cells were rotated for 30 minutes at 4°C. Lysates were sonicated a further 2 times at 30% amplitude for 20 seconds. Lysates were centrifuged at 16,000 x g in a Beckman JA-25.50 rotor for 20 minutes at room temperature. Soluble protein fraction was filtered through a 0.2 µm filter before being added to 1ml of pre-washed Amylose Resin (New England BioLabs) in a 50 ml tube. Protein batch binding was carried out for 2 hours at 4°C with rotation. Resin was

washed 3 times with 20ml of 20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.4 supplemented with Complete EDTA Protease inhibitor cocktail (Roche) by centrifugation at 2,000 x g in a swinging bucket rotor for 4 minutes at 4°C. Beads were transferred to 2ml low adhesion tubes and protein was eluted by incubation with 20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.4 supplemented with Complete EDTA Protease inhibitor cocktail (Roche) and 0.1mM Maltose for 2 hours at 4°C.

3.0 Geminin is Stable and Inactive in Interphase *Xenopus* Egg Extracts

3.1 Introduction

Geminin was originally identified as a protein that when expressed in *Xenopus* egg extracts was destroyed by the APC/C (McGarry and Kirschner, 1998). *Xenopus* egg extracts contain large stockpiles of proteins that function during the cell cycle, allowing them to undertake the cell cycle in the absence of transcription. Since geminin was found to be a target of the APC/C and was shown to be absent from somatic human cells in G1 (McGarry and Kirschner, 1998, Wohlschlegel et al., 2000), it was expected that endogenous geminin would be absent from *Xenopus* interphase extracts. However, it was found that a proportion of geminin remains stable in egg extracts and becomes the main nucleoplasmic inhibitor of rereplication (Hodgson et al., 2002, Arias and Walter, 2005). This implies that not only is a portion of the endogenous protein stable in interphase, but that this stability is essential to ensure once per cycle DNA replication. Importantly, it was then shown that this observation is not specific to the *in vitro* system and the levels of geminin do not vary significantly *in vivo* during the cleavage cycles of the *Xenopus* early embryo (Kisielewska and Blow, 2012). In addition, evidence is accumulating to suggest that this is a property of all embryonic stem cells (Quinn et al., 2001, Yang et al., 2011).

This project was undertaken using *Xenopus* egg extracts with the aim of elucidating the mechanism of geminin inactivation. The addition of calcium to metaphase arrested extracts triggers the metaphase-anaphase transition and cell cycle entry and this is accompanied by an abrupt activation of the licensing system. To enable a study of the events that occur shortly after calcium stimulation that lead to the activation of

licensing, the majority of experiments were carried out using metaphase-arrested extracts. *Xenopus* egg extracts provide a robust system for the study of DNA replication, however, there can be subtle but significant differences in the activities and properties of extracts prepared by different protocols in different laboratories. One relevant example of this was the initial controversy that followed the observation that geminin is in fact stable in interphase egg extracts. This issue was resolved when it was shown by multiple laboratories that geminin is the main nucleoplasmic inhibitor of rereplication in egg extracts and also stable *in vivo* during the cleavage cycles of the early embryo.

Thus it was of vital importance to confirm the various reports that indicate that geminin is both stable and inactive following the metaphase-anaphase transition, and to ensure that extracts prepared for use during this project supported the reported functions. This would show that metaphase-arrested extracts are an appropriate system to facilitate a characterisation of the mechanism of geminin inactivation.

3.2 Destruction kinetics of Geminin and Cdt1

In order to demonstrate the stability of geminin and Cdt1 after the metaphase-anaphase transition long timecourses were undertaken in the absence of added DNA. Under these conditions, only APC/C dependent destruction should be observed. Samples were taken at intervals after the addition of calcium to metaphase-arrested extracts and geminin and Cdt1 protein levels were analysed by western blot and quantified by 2D densitometry (Figure 8).

The result from two extracts demonstrate that there is some degree of extract to extract variation in the exact amounts of protein destroyed, however the kinetics of destruction for geminin and Cdt1 are reproducible (Figure 8). There is a clear difference in the

kinetics of Cdt1 and geminin destruction and this was the case in all extracts tested. Approximately 50-60% of geminin is degraded within 10 minutes after the addition of

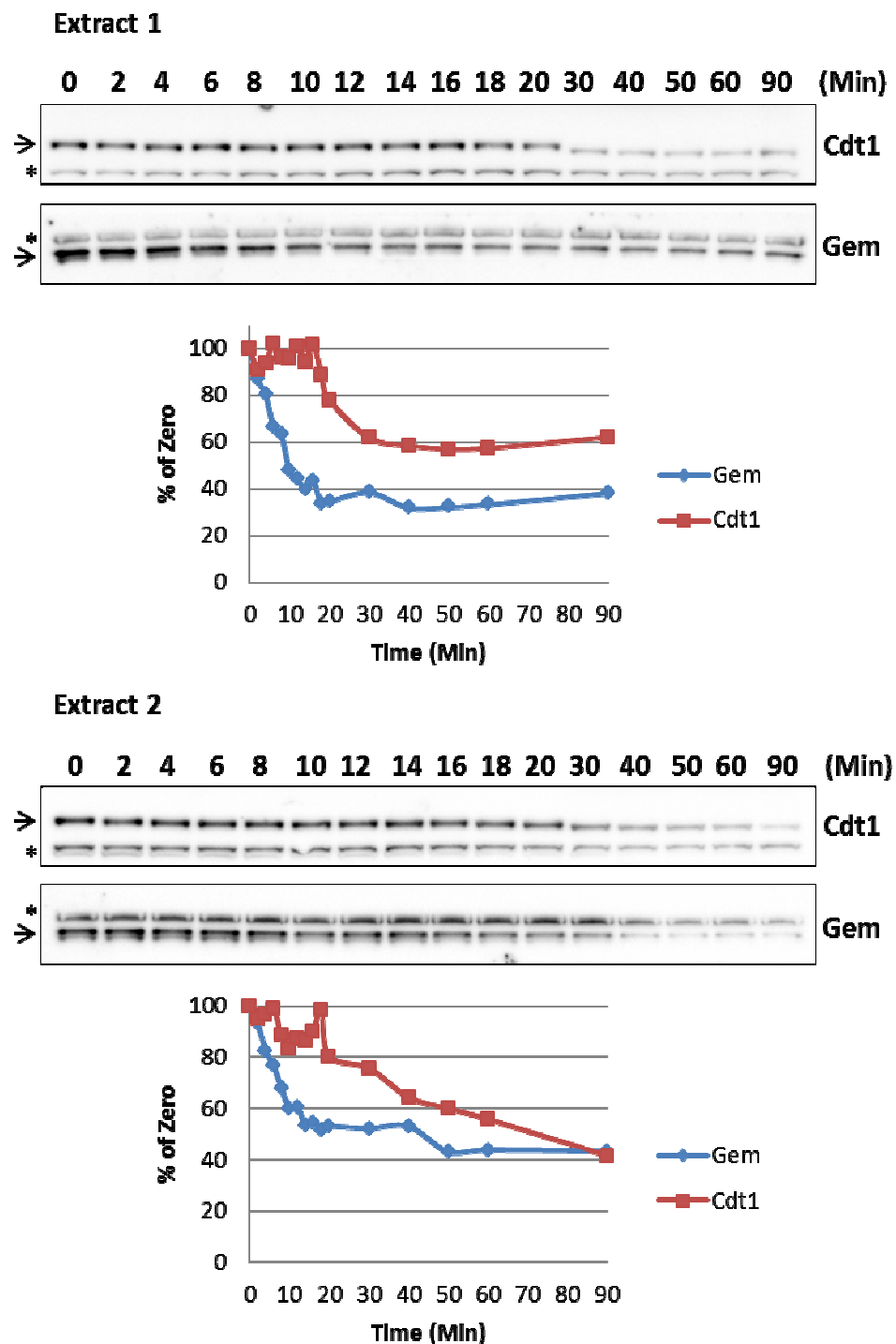


Figure 8: 40-50% of Geminin is stable 90 minutes after entry into Interphase. Samples were taken at different times after the addition of calcium and analysed by western blot to demonstrate the stability of geminin and Cdt1. Bands were quantified and plotted as a percentage of zero. Two extracts are shown to demonstrate extract to extract variation in the total levels of destruction at 90 minutes. Arrows indicate specific bands. * represents non-specific bands.

calcium. The remaining 40% of the protein remains stable thereafter. Geminin contains a ubiquitin initiation motif making it an efficient target of the APC/C (Williamson et al., 2011), which is likely to be responsible for the rapid destruction observed. Within the initial 10 minutes of high geminin instability Cdt1 levels remain relatively constant. Cdt1 destruction occurs later, beginning after approximately 20 minutes. Cdt1 in somatic cells is a target of the APC/C^{Cdh1} (Sugimoto et al., 2008). Cdh1 is not expressed in *Xenopus* until later in development; therefore all APC/C activity in extracts depends on APC/C^{Cdc20} (Lorca et al., 1998). This may account for the slow kinetics of Cdt1 destruction, where efficient targets of APC/C^{Cdc20}, such as securin, Cyclin B and geminin would be targeted first followed by a second wave of targets such as Cdt1. The observation that geminin levels remain approximately constant during the period when Cdt1 is being degraded suggests that the surviving geminin is in some way protected from the APC/C-mediated degradation.

Whereas geminin destruction follows a hyperbolic curve where it is rapidly degraded followed by a period of relative stability, after an initial lag Cdt1 destruction follows a slow linear reduction in levels. However, the extent of Cdt1 destruction varied between extracts. This experiment clearly demonstrates that a portion of geminin remains stable after metaphase-arrested extracts pass through the metaphase-anaphase transition into interphase. After an initial period of rapid destruction, approximately 50% of geminin remains stable during the licensing period and over time levels slowly fall and stabilise at a level of 30-40% by 90 minutes.

3.3 Destruction kinetics in the presence of DNA

Cdt1 is subjected to DNA replication-dependent destruction and the extent of destruction depends on the amount of DNA added (Arias and Walter, 2005, Kisielevska and Blow, 2012). It has been shown that geminin levels remain high *in vivo* (Kisielevska and

Blow, 2012), however the effect of DNA concentration on geminin stability has not been investigated in extracts. After fertilisation *in vivo*, the concentration of DNA during the first cell cycle is very low, approximately 10 pg/ μ l, therefore the kinetics of Cdt1 destruction shown in the prolonged timecourse above are not likely to differ significantly from the kinetics of destruction *in vivo*. However, in the absence of DNA, licensing, nuclear envelope assembly and replication do not occur. Therefore, it was also of interest to determine the stability of geminin and Cdt1 in the presence of DNA throughout the licensing period and during DNA replication to determine if geminin remains stable over long timecourses in the presence of DNA, as occurs *in vivo*.

The concentrations of DNA added were 3 and 20 ng/ μ l which represent approximately the DNA concentrations at cleavage cycles 8 and 11 respectively. DNA replication was measured to show that extract was capable of replicating up to 25 ng/ μ l of DNA (Figure 9A). DNA replication timecourses were performed and samples were taken at different times post calcium addition, analysed by western blot and quantified by 2D densitometry. Egg extracts, in general, are very viscous solutions making the equal pipetting of small volumes technically challenging. The viscosity also changes as extracts progress through the cell cycle, becoming more viscous over time. To minimise this effect and to ensure samples were equally loaded cut-off P2 pipette tips were used allowing equal loading in the absence of DNA. However the presence of DNA exacerbated this issue and some variability in loading was unavoidable; this can be seen by assessing at the intensity of non-specific bands (Figure 9B). Therefore to more accurately quantify the western blots by 2D densitometry, intensities were normalised against the non-specific bands. An additional timepoint of 120 minutes was included to show levels at the end of S-phase. A control containing no DNA gave the same result as previously shown (Figure 8), with geminin destruction kinetics following a hyperbolic

curve and Cdt1 remaining stable for 20 minutes before a slow linear decrease in levels. The addition of 3ng/ μ l DNA resulted in a greater reduction of Cdt1 levels compared to

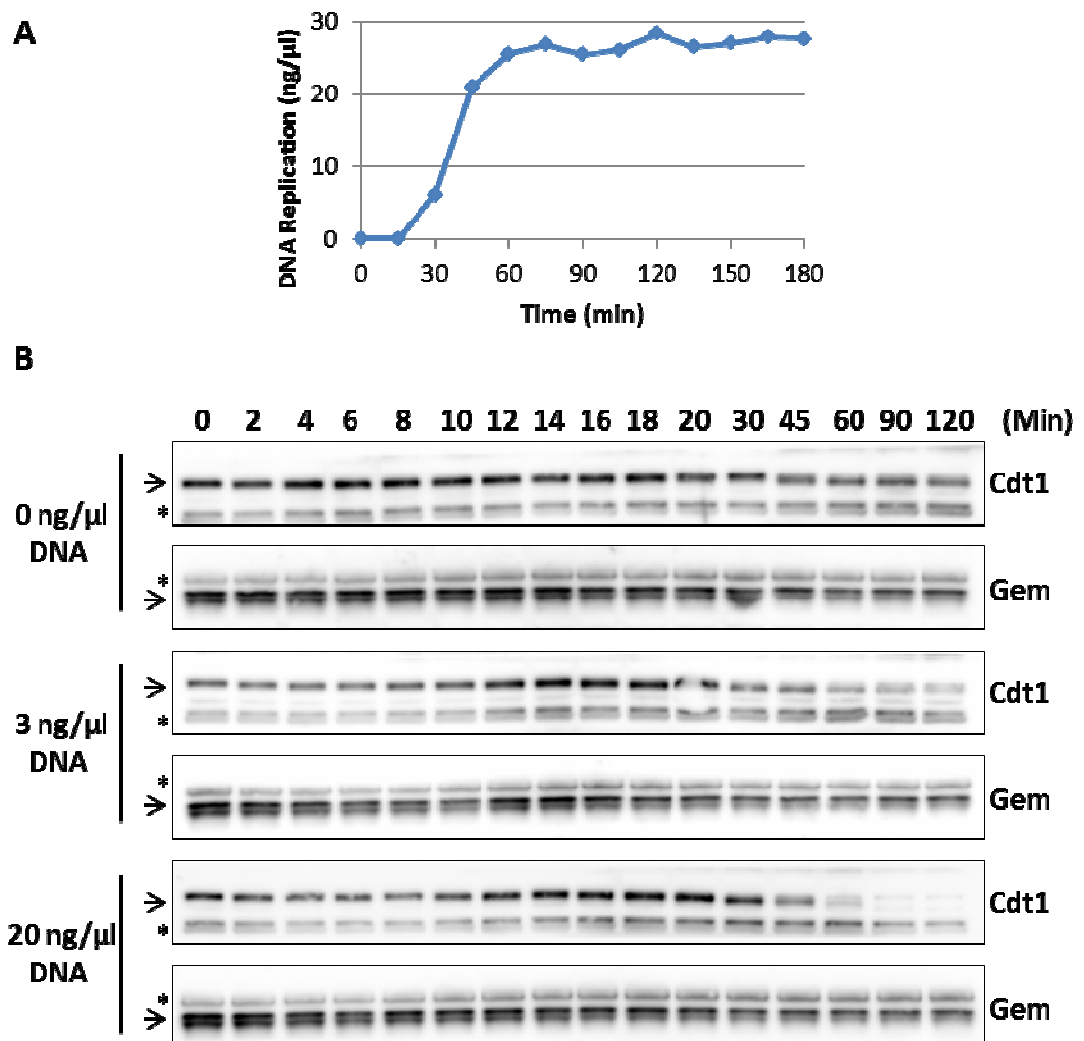


Figure 9: The Kinetics of Geminin and Cdt1 Destruction in the Presence of DNA. (A) To demonstrate the capacity of the extract a DNA replication assay was carried out at 25 ng/ μ l of DNA. (B) Extracts were supplemented with 0/3/20 ng/ μ l of DNA and incubated for 5 minutes prior to calcium addition. Samples were taken at different times after the addition of calcium and analysed by western blot to demonstrate the stability of geminin and Cdt1. Arrow indicate specific bands. Non-specific bands are denoted by an asterisk. Samples between 12-20 minutes were reproducibly overloaded, therefore intensities measured by 2D densitometry were normalised to nonspecific bands.

the no DNA control, with levels beginning to stabilise at later timepoints. This later stabilisation compared to the no DNA control may be due to the sequestration of Cdt1 in the nucleus where the APC/C is inactive, whereas in the absence of DNA the APC/C remains weakly active and continues to degrade Cdt1. The addition of 20ng/ μ l results in

an almost complete destruction of Cdt1. The Cdt1 destruction observed is DNA replication-dependent (Arias and Walter, 2005, Arias and Walter, 2006) and the timing is concurrent with the initiation of DNA replication during this experiment (Figure 9A). As an additional control to show that extracts had entered interphase, samples were taken at 120 minutes and nuclei were observed by UV/Phase microscopy. Large round nuclei were present with recondensed DNA, indicative of G2 extracts which have replicated their DNA. The addition of DNA at either concentration did not have any effect on the stability of geminin, compared to the no DNA control. Geminin destruction followed a hyperbolic curve, as observed in all samples, with the majority of geminin destruction occurring within 10 minutes of calcium addition followed by stabilisation of 40-50 % of protein (Figure 9B). Geminin degradation was largely unaffected by the addition of DNA.

The effect of DNA on Cdt1 stability can be seen when comparing the quantified data (Figure 10). Cdt1 degradation increases with DNA concentration as expected. However, the addition of DNA had no effect on the amount or kinetics of geminin destruction (Figure 10). This clearly demonstrates that geminin remains stable after extracts enter the cell cycle after metaphase arrest. In extracts DNA becomes maximally licensed within 15 minutes of calcium addition. Here, greater than 50% of endogenous geminin is present throughout the licensing period of 15 minutes, and this seemingly has no effect on the ability to replicate DNA. This suggests that geminin has been inactivated for its ability to inhibit licensing.

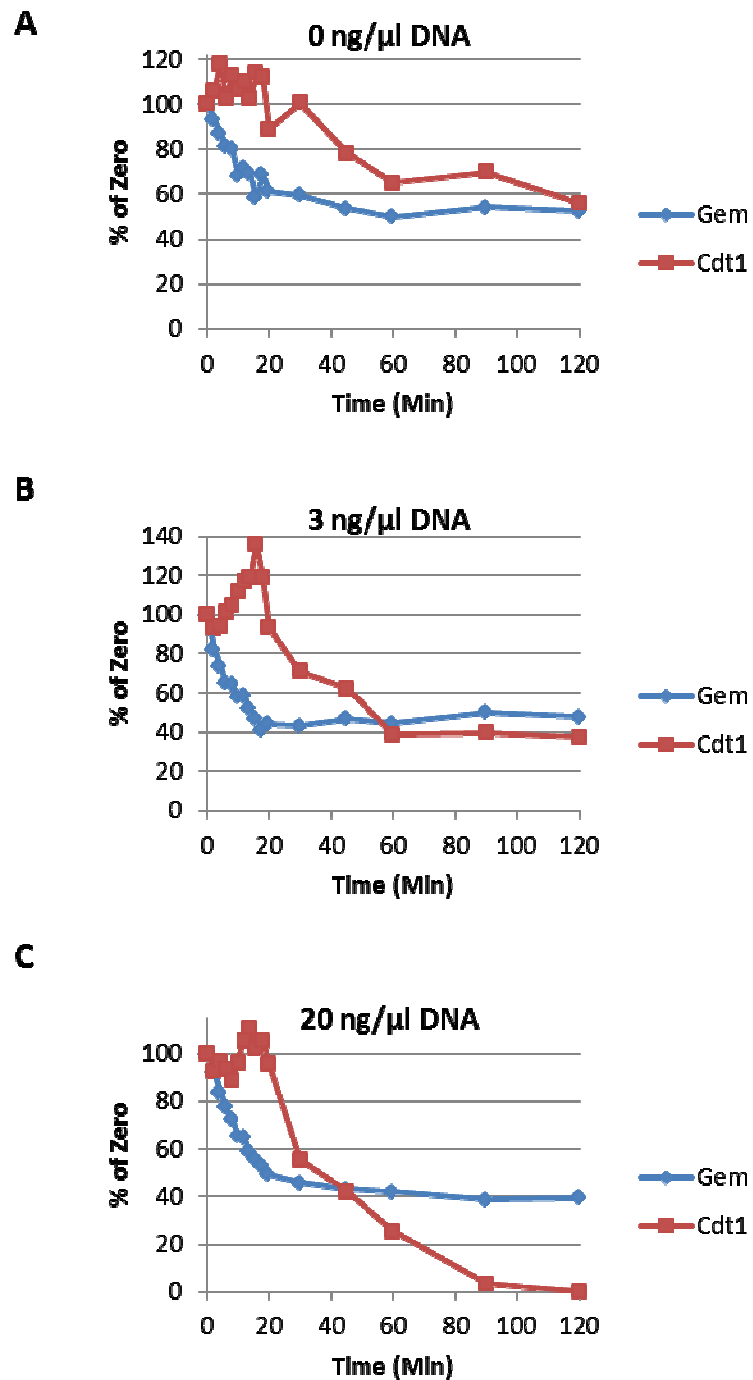


Figure 10: Geminin levels are unaffected by the presence of DNA. Samples shown in Figure 9B were quantified by 2D densitometry. Intensities were normalised against nonspecific bands and plotted as a percentage of 0 minutes. Cdt1 destruction increases with DNA concentration, whereas geminin destruction kinetics and total levels destroyed are unaffected.

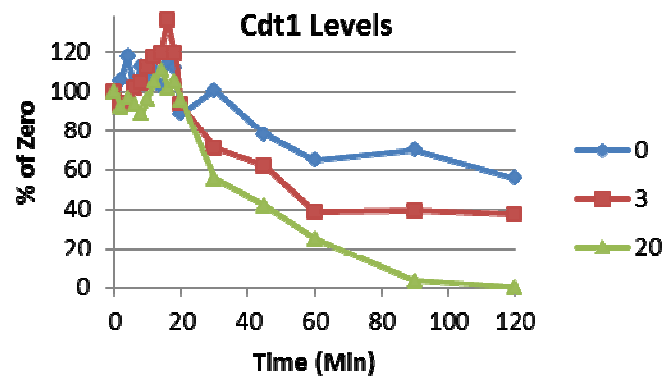
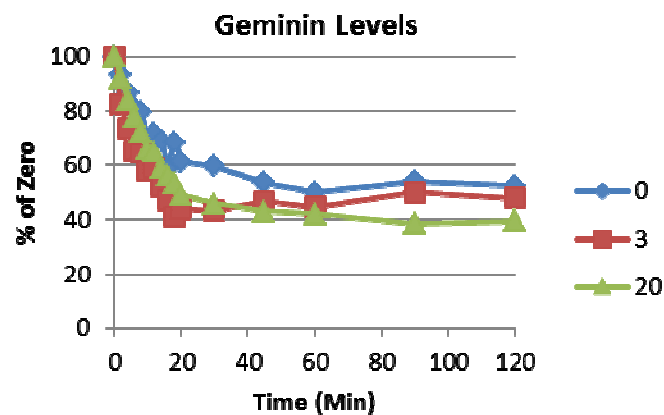
A**B**

Figure 11: The Effect of DNA concentration on Geminin and Cdt1 Stability. Data from Figure 10 is plotted together to show the effects of DNA addition on protein stability. Cdt1 is unstable in the presence of DNA due to replication coupled destruction. Geminin levels are unaffected by the addition of DNA.

3.4 Geminin-Cdt1 complexes break after the Metaphase-Anaphase Transition

Geminin is a potent inhibitor of DNA replication due to its ability to bind to and inhibit Cdt1 and in turn inhibit replication licensing. As shown above, *Xenopus* egg extracts can license and replicate DNA while in the presence of at least 50% of the normal pool of endogenous geminin which persists during the licensing period (Figure 8 and Figure 9). Since geminin is such a potent inhibitor of licensing and levels persist during the

licensing reaction, it is likely that geminin in interphase extracts is inactive. Evidence supporting this hypothesis has previously been published by our laboratory. Geminin inhibits Cdt1 by binding to it (Tada et al., 2001, Wohlschlegel et al., 2000). Therefore the most straightforward assay for geminin activity is to observe complex formation with Cdt1. Previous reports have shown that recombinant geminin^{DEL} (Geminin with a D-box deletion) can interact with interphase Cdt1, as determined by gel filtration (Li and Blow, 2004), while the majority of interphase geminin does not bind to recombinant Cdt1, as determined by Ni-NTA pulldown of His₆-Cdt1 (Hodgson et al., 2002). Taken together these data suggest that interphase geminin is not competent to bind to Cdt1 and is therefore inactive. It was important to further investigate these observations and show in my extracts prepared during this project that whereas interphase geminin is not competent to bind Cdt1, interphase Cdt1 is competent to bind geminin. This would be achieved by undertaking experiments that demonstrate protein-protein interactions while ensuring consistent methodologies to perform reciprocal experiments. There was a preference for endogenous protein-only experiments, as previous reports have suggested that recombinant geminin may be regulated differently than endogenous as recombinant was preferentially degraded, while endogenous was inactivated (Hodgson et al., 2002).

Geminin and Cdt1 were immunoprecipitated from metaphase and interphase extracts in order to observe their ability to interact in each cell cycle phase. Immunoprecipitation of endogenous geminin from metaphase extract pulled down high levels of Cdt1, demonstrating that there is a strong interaction between geminin and Cdt1, as expected (Figure 12). The reciprocal immunoprecipitation of Cdt1 gave a similar result, with a strong pulldown of geminin. This can be seen when comparing the intensity of geminin and Cdt1 in metaphase samples. However, immunoprecipitation of geminin from interphase extract resulted in a greatly reduced pulldown of Cdt1 (Figure 12, lanes 2 and

6). In addition, during reciprocal Cdt1 immunoprecipitates, geminin was only observed at very high exposures indicating that only a very weak interaction exists in interphase (Figure 12, lane 8). This demonstrates that the interaction of endogenous geminin and Cdt1 is dramatically reduced after the metaphase-anaphase transition. However, it is never a complete separation of geminin-Cdt1 complexes as some degree of co-immunoprecipitation of proteins remains in reciprocal experiments in interphase extracts.

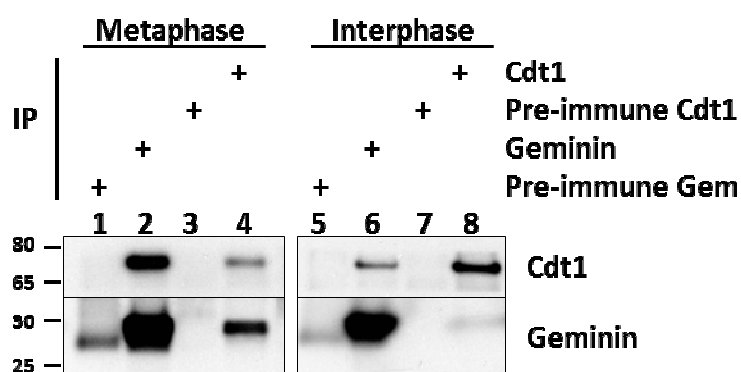


Figure 12: Geminin-Cdt1 Interactions Change Dramatically in Interphase. (A) Metaphase and interphase extracts were incubated with pre-immune serum or anti-geminin/Cdt1 serum on ice for 45 minutes. Samples were supplemented with 30% (v/v) protein A Sepharose. Beads were isolated, washed and samples were analysed by western blotting. (B) Metaphase and interphase extracts were supplemented with 100 nM His₆-Cdt1 for 30 minutes on ice. Samples were supplemented with 10 µl of Ni-NTA and incubated on a roller at 4°C for 30 minutes. Beads were isolated and washed and pulldowns were analysed by western blotting. (C) Same for (B) except samples were supplemented with 100 nM His₆-geminin^{DEL}.

To further investigate the change in the interaction of geminin and Cdt1 metaphase and interphase extracts were fractionated by size exclusion chromatography. This revealed a number of cell cycle phase-dependent complexes. The fractions from two individual columns are shown to demonstrate any slight column-to-column variation in sample fractionation from 443-66 kDa. Samples from the void volume are also shown to demonstrate that there are no peaks higher than 669 kDa, consistent with previous

reports (Figure 13)(Hodgson et al., 2002, Li and Blow, 2004). In metaphase extracts, a high molecular weight complex of geminin and Cdt1 is observed between 669-443 kDa with an additional proportion of geminin fractionating into a separate peak, between 443-200 kDa (Figure 13)(Hodgson et al., 2002, Li and Blow, 2004). 15 minutes after the metaphase-anaphase transition in interphase extracts, Cdt1 runs at a lower molecular weight, consistent with the loss of its binding to geminin. During interphase, geminin fractionates into two previously unidentified peaks, one that partially co-fractionates with Cdt1 between 443-200 kDa and a second smaller peak between 200-66 kDa (Figure 13). These results differ from previous reports where only a single interphase peak was identified (Hodgson et al., 2002, Li and Blow, 2004). However, the current work used a MAbPac SEC-1 column which provides a greater resolution across the desired range of 669-69 kDa compared to the Superose 6 columns used in previous experiments. The fractions from two different columns are shown to demonstrate that there is a slight column to column variation in the resolution of the peaks. Comparing the migration of geminin in metaphase and interphase, the lower molecular weight metaphase peak and the higher molecular weight interphase peak partially overlap, but are shifted by a fraction (Figure 13A, lane 11 and 12; Figure 13B, lane 12), which could be a difference of 100 kDa at this column's resolution. This suggests there are four distinct geminin complexes, two in metaphase and two in interphase. These data show that the majority of high molecular weight metaphase geminin and Cdt1 complexes separate during exit from metaphase, further supporting the observation made by immunoprecipitation of endogenous proteins that geminin-Cdt1 interactions change significantly as extracts enter the cell cycle.

There was an issue with non-specific bands partially co-fractionating with geminin (Figure 13, lanes 15, 16 and 17. Figure 14) which also run close to geminin on SDS-PAGE. To show that this band is non-specific, geminin was immunodepleted and

extract was fractionated by size exclusion chromatography. Extract was >95% depleted of geminin compared to whole extract and a band can be seen in the depleted extract which is likely to correspond to the contaminating band found in gel filtration fractions (Figure 14A). The contaminating bands persisted and fractionated into the same fractions in the absence of geminin (Figure 14B, lane 8, 9 and 10), suggesting that it is indeed non-specific. These non-specific bands could also be separated from geminin on SDS-PAGE by running a 12% Bis-Tris gel (Figure 14C).

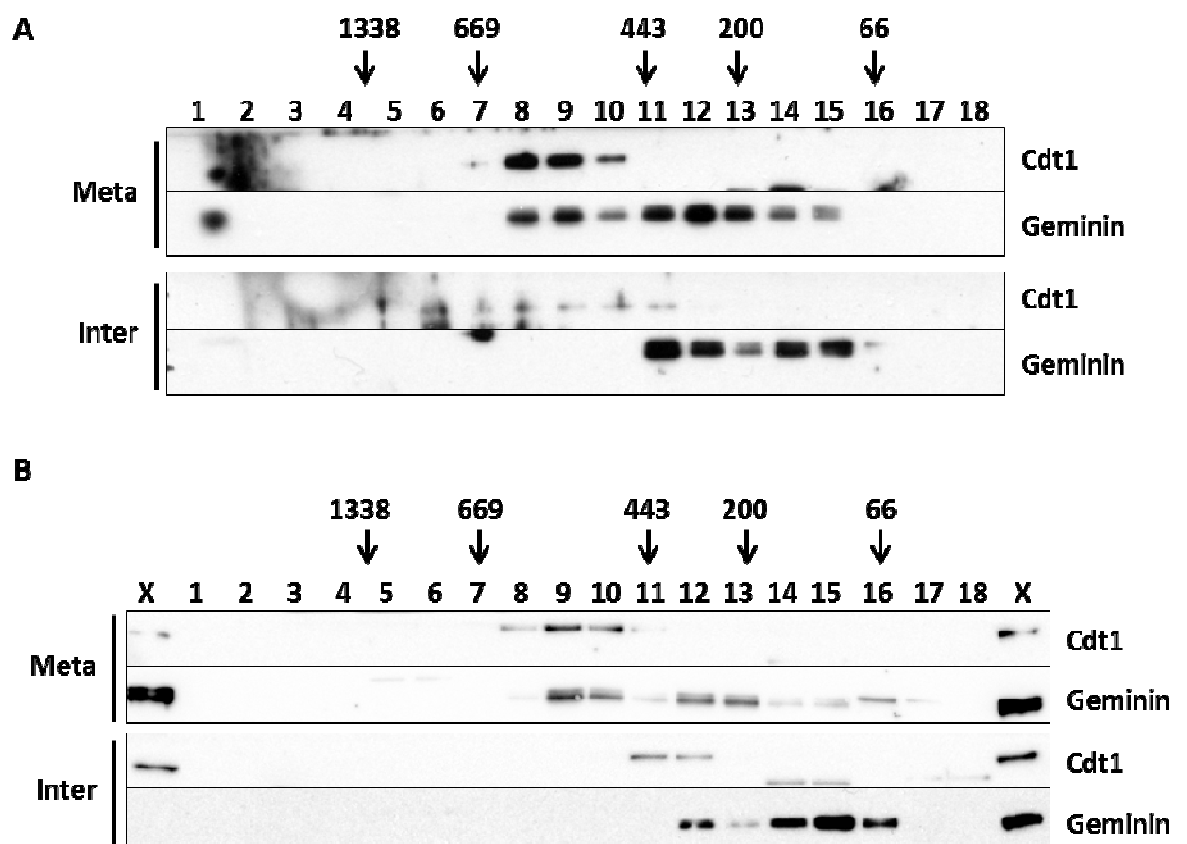


Figure 13: Geminin is fractionated into Multiple Peaks by Size Exclusion Chromatography. Metaphase or interphase extract was diluted 1:5 with LFB1/50 and spun through a 0.2 μ m filter. Samples were loaded at 1% of total column volume on a MAbPAC SEC-1 and fractionated at 0.15 μ l/min into 75 μ l fractions. 25 μ l of fractions were analysed by western blotting. (A) and (B) show the fractionation of two separate columns. X indicates input extract to show specificity of bands.

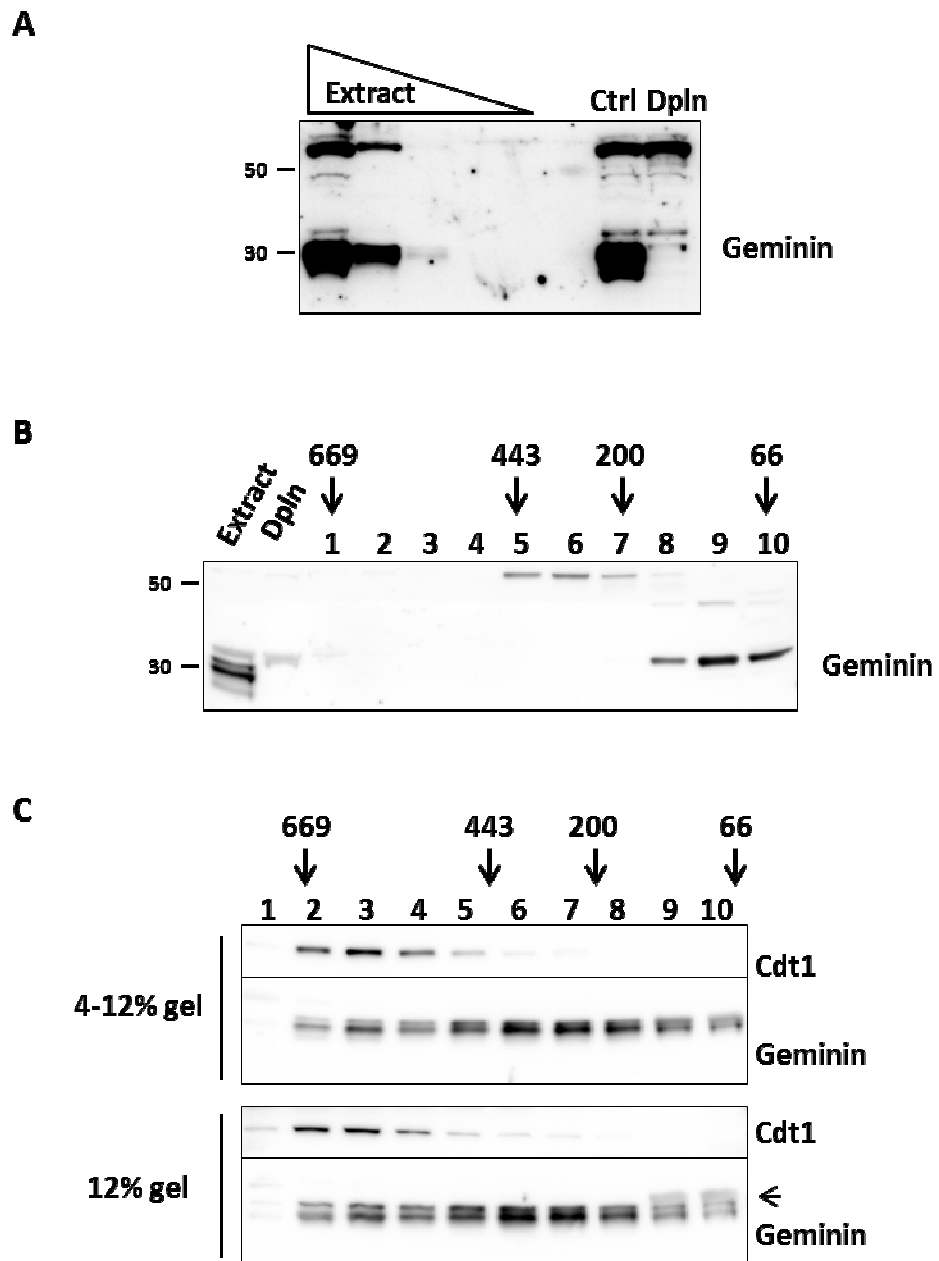


Figure 14: Specificity of Geminin Bands on Gel Filtration. (A) Extracts were immunodepleted of geminin. A titration of extract versus an equivalent amount of total and depleted extract were blotted for geminin to quantify the depletion. (B) Geminin immunodepleted extract was loaded at 1% of total column volume on a Mab PAC SEC-1 and fractionated at 0.15 μ l/min into 75 μ l fractions. 25 μ l of fractions were analysed by western blotting and key fractions were blotted to show the fractionation of non-specific bands which run close to geminin on SDS-PAGE in lanes 8, 9 and 10. (C) Extract was loaded at 4% of total column volume on a Mab PAC SEC-1 and fractionated as in (B). 25 μ l of fractions were separated by SDS-PAGE on either a 4-12% or 12% fixed Bis-Tris gel and analysed by western blotting. Arrowhead indicates the appearance of a non-specific band above geminin in fractions 8, 9 and 10.

3.5 Endogenous Interphase Geminin cannot bind recombinant Cdt1

To determine whether the loss of geminin-Cdt1 complex formation on exit from metaphase was dependent on changes to geminin or Cdt1, the interactions of endogenous Cdt1 or geminin with recombinant proteins was investigated. His₆-Cdt1 was incubated in metaphase and interphase extract for 30 minutes and recovered with Ni-NTA (Figure 15A). Geminin was specifically pulled-down with His₆-Cdt1 in metaphase extracts, showing that it is competent to bind to Cdt1 and is therefore active. A relatively low amount of geminin was pulled-down with His₆-Cdt1 from interphase extracts suggesting that the majority of geminin in interphase extracts is unable to bind to His₆-Cdt1. To show that this was not due to an activity in interphase extracts which would make His₆-Cdt1 incompetent to bind to geminin the reciprocal experiment was performed. His₆-Gem^{DEL} was incubated in metaphase or interphase extract for 30 minutes before being recovered with Ni-NTA (Figure 15B). His₆-Gem^{DEL} was used rather than full length geminin to ensure that the results could not be altered by ongoing APC/C-dependent destruction of the recombinant geminin. His₆-Gem^{DEL} recovered from metaphase did not pull down significant amounts of endogenous Cdt1 compared to a control containing noHis₆-Gem^{DEL}. This is likely because all of the endogenous endogenous Cdt1 is already bound to geminin, via a particularly strong interaction (Tada et al., 2001). In contrast, His₆-Gem^{DEL} recovered from interphase extracts yielded high levels of Cdt1. This implies that endogenous Cdt1 is competent to bind to geminin in interphase egg extracts.

These data suggest that the abilities of metaphase and interphase endogenous geminin differ in their ability to interact with Cdt1. To further pursue this observation, metaphase and interphase extracts were supplemented with 100 nM His₆-Cdt1 for 15 minutes

before being diluted 1/5 with buffer (LFB1/50), filtered through a 0.2 μ m filter and fractionated by size exclusion chromatography. His₆-Cdt1 added to metaphase extracts

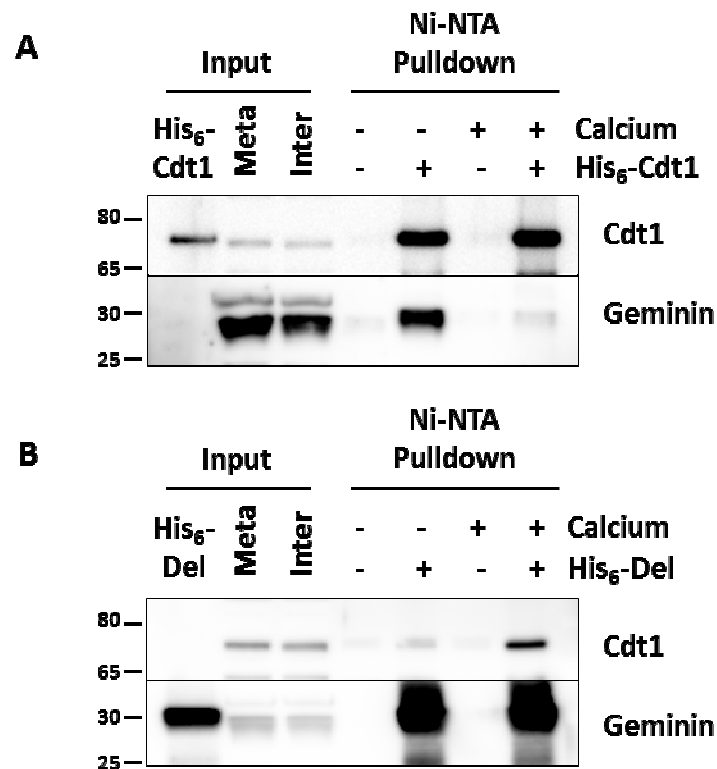


Figure 15: Interphase Geminin does not interact with His₆-Cdt1 by Pulldown. Metaphase or interphase extracts were supplemented with (A) His₆-Cdt1 or (B) His₆-Gem^{DEL} and incubated on ice for 30 minutes. Recombinant proteins were recovered with Ni-NTA. 25% inputs are shown.

bound to the excess geminin that normally runs at a peak of 200-400 kDa (Figure 16, lane 9 and 10), to form additional high molecular weight geminin-Cdt1 complexes between 443-669 kDa (Figure 16, lane 6 and 7). This implies that excess geminin in metaphase extracts is competent to bind to Cdt1, in agreement with observations from pulldown experiments (Figure 15A). However, His₆-Cdt1 added to interphase extracts had no effect on the migration of endogenous geminin (Figure 16B). Both geminin interphase peaks eluted in the same fractions as the interphase extract only control. This shows that endogenous interphase geminin is not competent to bind to Cdt1, again agreeing with pulldown experiments.

The converse experiment was carried out to show that the loss of interaction was specific to geminin and not a change in the ability of Cdt1 to be bound by geminin. Metaphase and interphase extracts were supplemented with 100 nM His₆-Gem^{DEL} for 15

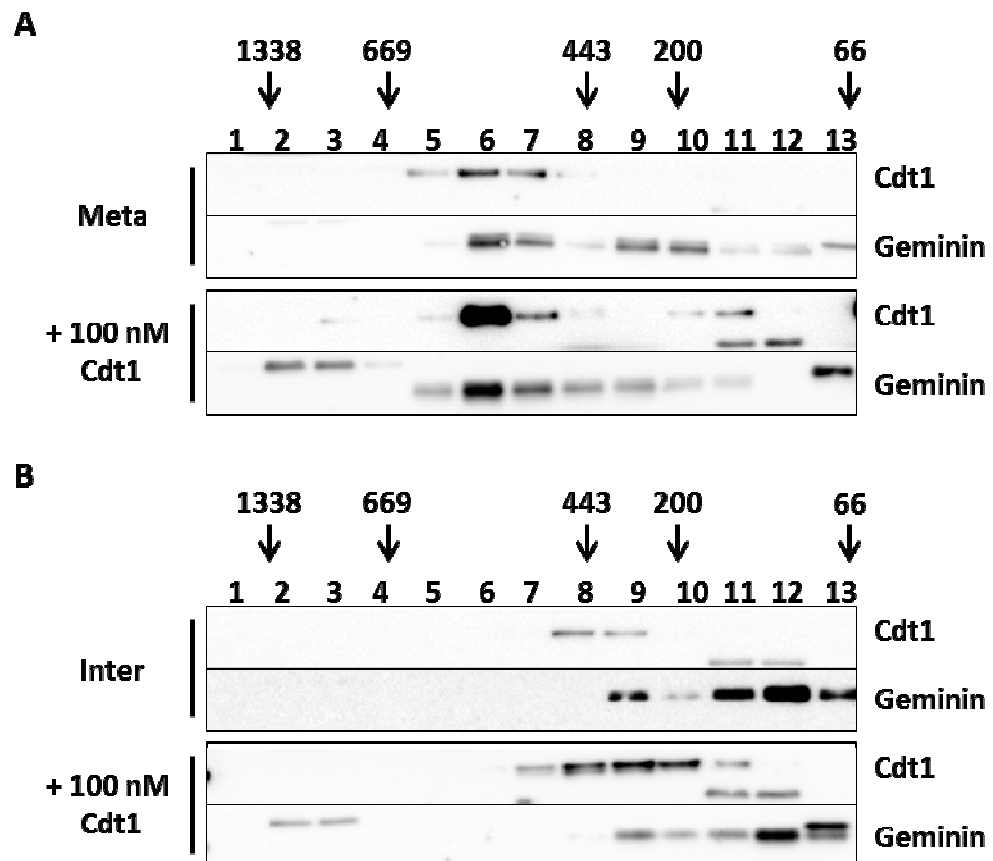


Figure 16: Interphase Geminin does not Cofractionate with His₆-Cdt1 by Size Exclusion Chromatography. (A) Metaphase extract was supplemented with 100 nM His₆-Cdt1 and incubated for 15 minutes. Extract was diluted 1:5 with LFB1/50 and filtered through a 0.2 µm filter before being fractionated by size exclusion chromatography. Fractions were analysed by western blotting to observe complex formation. (B) Interphase extract was treated as in (A).

minutes before being diluted 1/5 with LFB1/50, filtered through a 0.2 µm filter and fractionated by size exclusion chromatography (Figure 17). His₆-Gem^{DEL} added to metaphase extract fractionated into the Cdt1-free geminin peak between 443-200 kDa. This agrees with the observation that His₆-Gem^{DEL} does not pull down significant amounts of Cdt1 in metaphase. This shows that Cdt1 is maximally bound by

endogenous geminin in metaphase. The amount of geminin bound cannot be increased or exchanged with recombinant protein. His₆-Gem^{DEL} added to interphase extract could bind Cdt1 to reform the 669-443 kDa high molecular weight complex. This demonstrates further that Cdt1 in interphase extracts is capable of interacting with geminin, and that it is endogenous geminin that has been altered resulting in a loss of geminin-Cdt1 interactions.

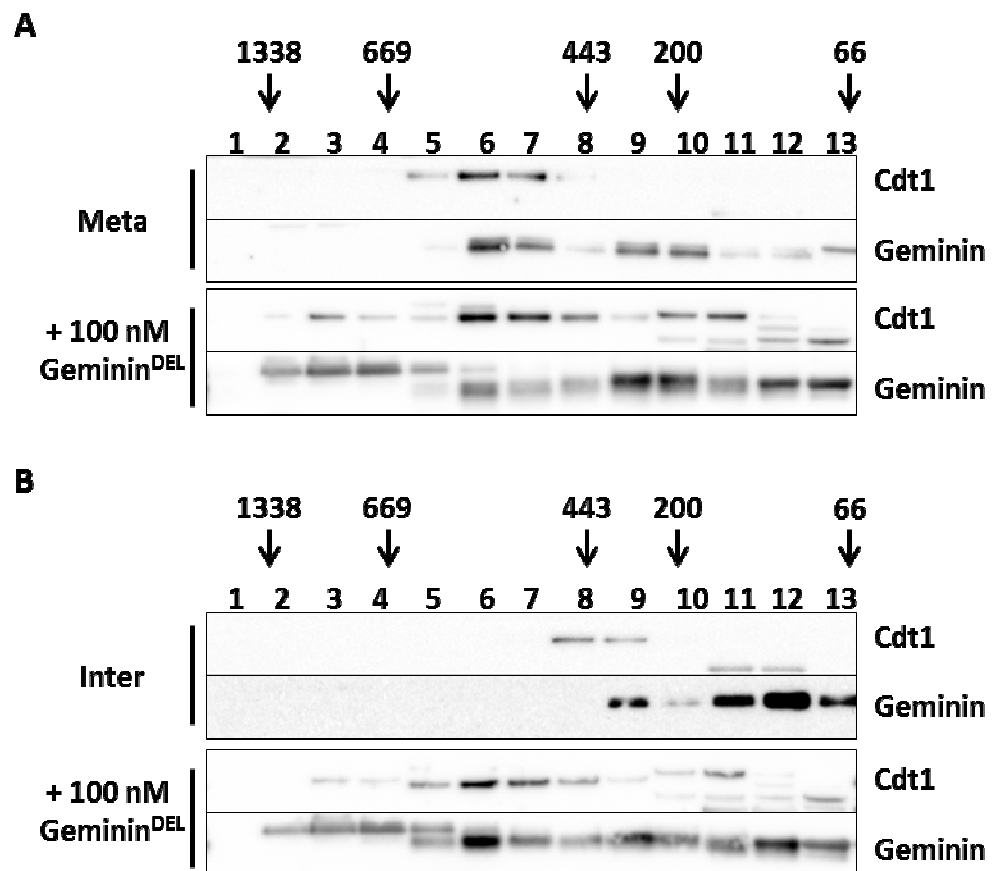


Figure 17: InterphaseCdt1Cofractionates with His₆-Geminin^{DEL} on Size Exclusion Chromatography.(A) Metaphase extract was supplemented with 100 nM His₆-Geminin^{DEL} and incubated for 15 minutes. Extract was diluted 1:5 with LFB1/50 and filtered through a 0.2 µm filter before being fractionated by size exclusion chromatography. Fractions were analysed by western blotting to observe complex formation. (B) Interphase extract was treated as in (A).

3.6 Summary - Geminin is Stable and Inactive in Interphase Extracts

Experiments carried out here have shown that geminin is a relatively stable protein in interphase extracts and up to 30-40% remains for at least 90 minutes. The addition of high levels of DNA results in the degradation of Cdt1, but has no effect on the stability of geminin. Greater than 50% of geminin remained in extract during the licensing reaction, and this did not interfere with the ability to replicate DNA. Therefore this interphase geminin does not interfere with the replication licensing reaction. This suggests that geminin is inactive in interphase egg extracts. The most simple assay that can be used to assess geminin function is to determine whether it can bind to Cdt1. Experiments here show that the interaction between geminin and Cdt1 changes dramatically after the metaphase-anaphase transition. In metaphase geminin exists in two major forms: in a high molecular weight complex with Cdt1 between 669 and 443 kDa and a lower Cdt1-independent 200 kDa complex. In interphase geminin also exists in two major forms: one at approximately 200 kDa that partially co-fractionates with Cdt1 and a lower Cdt1-independent complex that runs above 66 kDa. Assessing the interaction of geminin and Cdt1 with recombinant proteins has shown that the loss of interaction between geminin and Cdt1 is due to a change in geminin activity rather than Cdt1.

4.0 Regulation of Geminin by the APC/C and Post-Translational Modification

4.1 Introduction

The E3 ubiquitin ligase activity of the APC/C orchestrates the exit of *Xenopus* eggs from metaphase of meiosis II. To maintain a metaphase arrest eggs contain an APC/C inhibitory activity termed cytostatic factor (CSF). CSF activity is provided by Emi2 which inhibits the APC/C by protein-protein interactions to prevent ubiquitin ligase activity (Sako et al., 2014). A signalling cascade after calcium addition leads to the activation of CamKII and subsequently to the Plk- and SCF-mediated inactivation and destruction of Emi2 (Hansen et al., 2006). Loss of CSF results in the rapid activation of the APC/C. During metaphase arrest, licensing activity is inhibited by geminin-dependent inhibition of Cdt1 and to some extent by CDK activity (Tada et al., 2001). CDK activity is also required for APC/C activation thereby creating a negative feedback loop. CDKs activate the APC/C, which in turn destroys B-type cyclins leading to the inactivation of CDKs and the subsequent exit from metaphase (Li and Blow, 2004). A previous report has shown that APC/C activity is also essential for geminin inactivation (Li and Blow, 2004). Therefore, in addition to mediating mitotic exit (by destroying cyclin B), the APC/C also coordinates licensing activation by regulating the two inhibitors, geminin and CDKs (Figure 18).

Cyclin B is completely destroyed by APC/C-mediated ubiquitination in egg extracts. This destruction means that *Xenopus* egg extracts must translate new protein in order to pass through multiple cell cycles, as cyclin B protein must be translated for mitotic entry at the end of each cycle. Geminin, however, is not completely destroyed (Hodgson et al., 2002)(Figure 11) and interphase inactive geminin runs at its native molecular

weight on SDS-PAGE(Li and Blow, 2004). This observation led Li and Blow to propose that ubiquitination of geminin may be a prerequisite for its inactivation and while interphase geminin becomes deubiquitinated and in turn inactivated by some additional mechanism such as PTMs or by forming other protein-protein interactions (Li and Blow, 2004).

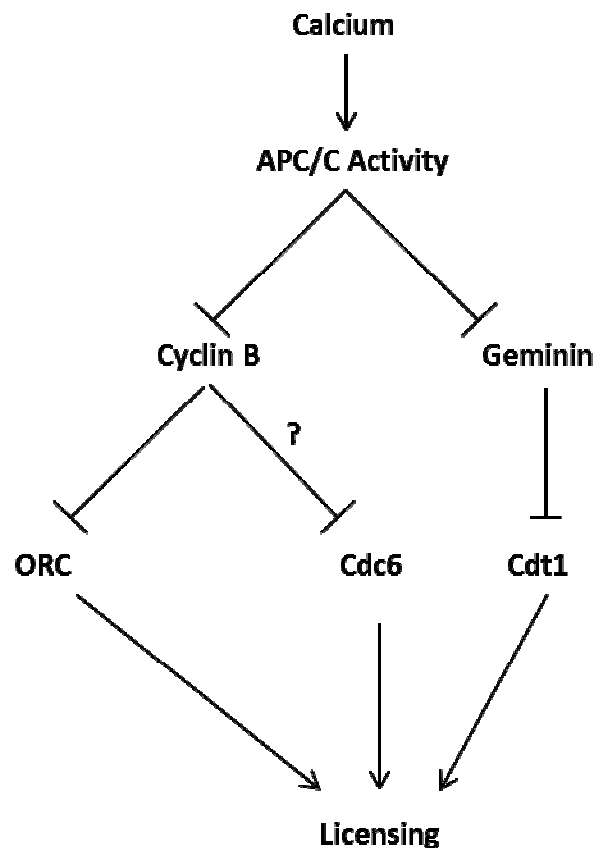


Figure 18: Control of Licensing activation by the APC/C. Geminin and CDK activity inhibit licensing activity in metaphase. Geminin inhibits Cdt1 by direct binding. CDK inactivates ORC by direct phosphorylation. It is not clear whether CDK inhibits Cdc6 in metaphase arrested egg extracts directly by phosphorylation or indirectly by preventing ORC chromatin binding. Activation of the APC/C by calcium addition leads to the rapid inactivation of CDK activity due to a complete degradation of B-type cyclins. Geminin inactivation is downstream of APC/C activity and it has been proposed that geminin is ubiquitinated prior to inactivation. APC/C-dependent inactivation of CDK and geminin triggers licensing activity.

It has been reported that inactive interphase geminin becomes reactivated after nuclear import in *Xenopus* egg extracts: following nuclear assembly a metaphase-like high molecular weight complex of geminin and Cdt1 is found on gel filtration (Hodgson et

al., 2002). This observation leads one to hypothesise a mechanism where geminin is regulated by post-translational modification. In this model, the activation of a geminin modifying protein at the metaphase-anaphase transition facilitates the activation of licensing in the cytoplasm. Licensed DNA then drives nuclear formation and the subsequent nuclear import of geminin. Separation of nuclear geminin from the inactivating activity of the cytoplasm or compartmentalisation with a demodifying protein leads to the reactivation of geminin to inhibit licensing before the initiation of DNA replication (Gillespie et al., 2007).

Multiple mechanisms have been hypothesised to account for geminin inactivation, a number of which involve post translational modification of geminin. The aim of this chapter is to gain insights into the molecular events that occur downstream of APC/C activation that result in the inactivation of geminin and to determine whether geminin activity is regulated by post translation modification.

4.2 The APC/C Mediates a Switch in Licensing Activity

A published report showed that geminin was subjected to APC/C-dependent ubiquitination and that this was a prerequisite for geminin inactivation (Li and Blow, 2004). Therefore it was necessary to investigate the role of the APC/C as it is known to be upstream of geminin inactivation and determine the effect of APC/C activation on geminin activity.

It has previously been shown that the 26S proteasome is dispensable for the activation of replication licensing. This was demonstrated using the well-established 26S proteasome inhibitor MG132 (Li and Blow, 2004). MG132 is soluble in DMSO and is required at relatively high concentrations for a complete inhibition of the proteasome in egg extracts. High amounts of DMSO causes problems with nuclear formation, therefore an alternative inhibitor was found. 0.1 mM bortezomib prevents cyclin B

destruction up to 15 minutes after the metaphase-anaphase transition, showing that at this concentration the proteasome is inhibited (Moreno et al., 2014). Replication licensing and DNA replication are unaffected by 26S proteasome inhibition, despite the fact that the licensing inhibitors geminin and cyclin B are not destroyed (Li and Blow, 2004). To show that geminin inactivation is unaffected by inhibition of the proteasome, metaphase extract supplemented with or without 0.1 mM bortezomib was released into interphase for 15 minutes and fractionated by size exclusion chromatography (Figure 19). The fractionation of inactive interphase geminin was unaffected by bortezomib addition, showing that protein destruction is dispensable for the inactivation of geminin. It has been reported that cyclin B destruction is not essential for CDK inactivation (Nishiyama et al., 2000, Chesnel et al., 2006). Therefore it is likely that geminin can also be regulated by methods other than proteolysis following APC/C dependent ubiquitination.

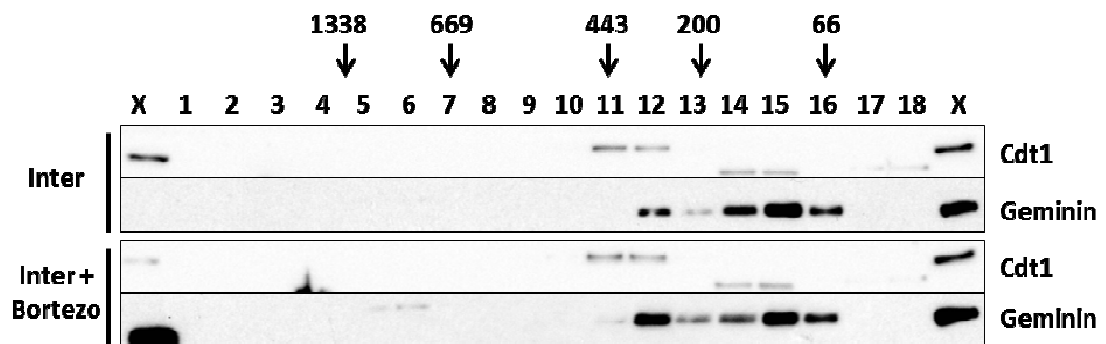


Figure 19: Protein Degradation is not required to Inactivate Geminin. Metaphase extract or metaphase extract supplemented with 0.1 mM bortezomib was released into interphase for 15 minutes before 1/5 dilution with LFB1/50, filtered through a 0.2 µm membrane and fractionated by size exclusion chromatography.

To assess the role of the APC/C in the activation of replication licensing, a specific APC/C inhibitor was required. A peptide of the cyclin B D-box motif (RRTALGDVTNKVSE) is a well-established competitive inhibitor of the APC/C used in *Xenopus* egg extracts (Peter et al., 2001). A D-box peptide was custom synthesised

and control experiments were undertaken to demonstrate the ability of the peptide to inhibit the APC/C. Metaphase extracts were supplemented with D-box peptide and Cdt1, cyclin B and geminin levels were measured by western blot analysis 30 minutes after the addition of calcium (Figure 20A). D-box peptide inhibited protein degradation at a concentration of 2 mM. A complete inhibition of the APC/C would render extracts incapable of metaphase exit in response to calcium addition. To demonstrate that this is the case in D-box treated extracts, nuclear formation was observed by UV/phase contrast microscopy after the addition of calcium. D-box treated extract failed to exit mitosis, as judged by a lack of nuclear formation after 30 minutes (Figure 20B). Taken together these data demonstrate that the APC/C is strongly inhibited by 2 mM D-box, in agreement with previous results generated using a different peptide (Li and Blow, 2004).

Since protein destruction is not required for the activation of licensing it is likely that APC/C activity directs inactivation of geminin and CDK through methods other than ubiquitin-mediated proteolysis. If a regulatory role such as this is mediated by the APC/C, is continuous APC/C activity required to maintain the ability to license? D-box peptide was used to gain more information about the length of time that APC/C activity is required for following its activation by calcium addition. An assay was undertaken to determine if continuous or ongoing APC/C activity is required after calcium addition to inhibit geminin and maintain the ability to replicate DNA. D-box peptide was added at defined times after calcium stimulation and DNA replication was measured after 90 minutes (Figure 21A). Addition of D-box peptide within 10 minutes of calcium addition resulted in a complete inhibition of DNA replication. This suggests that APC/C activity is essential for at least 10 minutes after calcium addition.

A

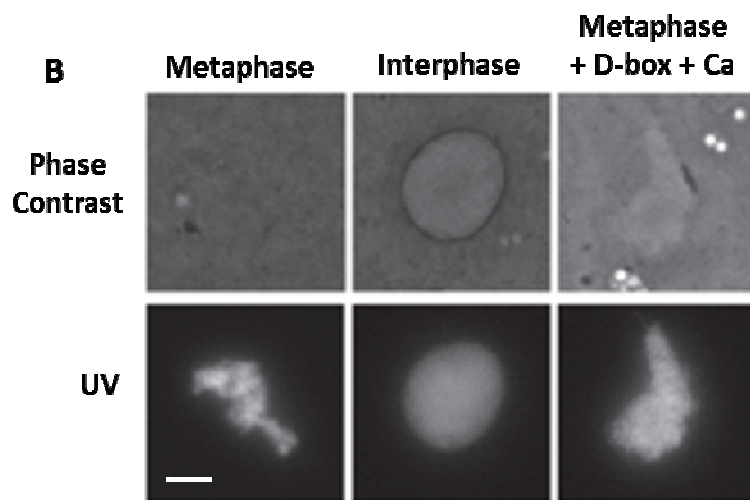
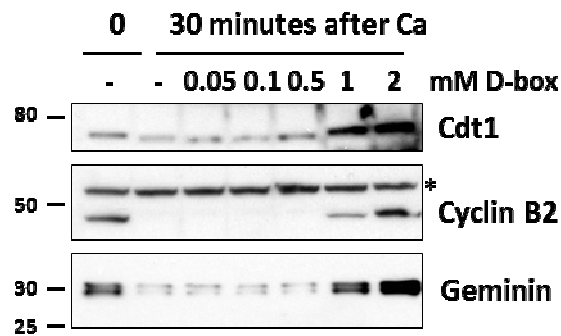


Figure 20: D-box peptide Inhibits APC/C activity. (A) D-box peptide was titrated into metaphase extract prior to calcium addition. The levels of APC/C targets Cdt1, geminin and Cyclin B2 after 30 minutes were analysed by western blot to determine the activity of the APC/C. (B) UV/Phase microscopy of sperm DNA incubated for 30 minutes in various extracts. Addition of 2 mM D-box before calcium addition prevents release from metaphase arrest, as DNA remains condensed and nuclei fail to form.

To determine whether the APC/C mediated event that occurs after 10 minutes is maintained or reversed in the absence of APC/C activity, D-box was added 10 minutes after calcium and extracts were incubated for defined times before the addition of DNA (Figure 21B). A loss of DNA replication after a prolonged incubation would suggest that the APC/C-dependent event is reversed over time. A reduction in DNA replication was observed after longer incubations, however this reduction also occurred in control samples, suggesting that the APC/C-dependent activity required for DNA replication is maintained after an initial 10 minutes of APC/C activity. Therefore, the APC/C

regulates DNA replication in a switch like manner. A threshold of APC/C activity is required and after this is reached APC/C activity is no longer required.

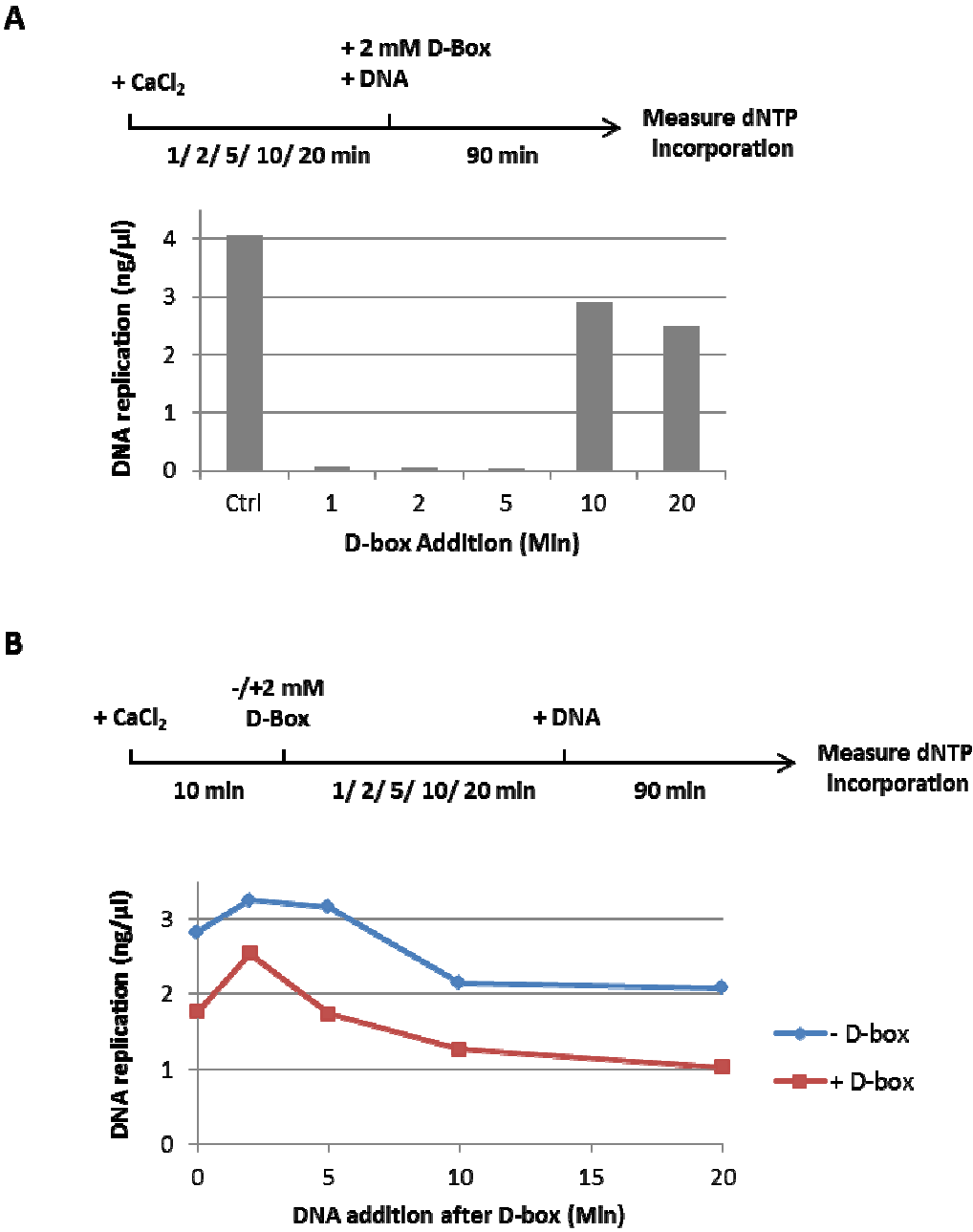


Figure 21: APC/C Activity is Required for 10 Minutes After Calcium Addition. (A) Extracts were supplemented with D-box at various times after calcium addition to inhibit the APC/C. DNA was added at the same time as D-box and DNA replication after a further 90 minutes was measured. (B) Extract was activated for 10 minutes before addition of either buffer or D-box. Extracts were incubated for defined times before the addition of DNA and DNA replication was measured at 90 minutes.

The assays above were an indirect measure of licensing, where it is inferred that if DNA replication occurred, then the DNA first must have been licensed. To confirm this, further experiments were designed to investigate the effects of APC/C inhibition on licensing activity more directly. To do this, assays included a minimal licensing step. ‘Maximally’ licensed DNA contains a vast excess of MCM2-7 complexes, which represent dormant origins. ‘Minimally’ licensed DNA has the minimum number of MCM2-7 complexes to support replication of ~100% of the input DNA with normal kinetics (Oehlmann et al., 2004, Woodward et al., 2006). In an extract where licensing is fully active, DNA requires a 3 minute incubation for minimal licensing of ~5 ng/μl of DNA, after which time further licensing activity can be inhibited by addition of geminin^{DEL}. By including a minimal licensing step in the D-box assay, the effect of D-box addition on licensing activity specifically can be measured. Assays were carried out where D-box or buffer was added at various times after calcium stimulation. DNA was subsequently added and incubated for 3 minutes before licensing was inhibited by the addition of geminin^{DEL}. Total DNA replication was quantified after 90 minutes (Figure 22). In control samples where extracts were supplemented with buffer only, a linear increase in licensing activity was observed which plateaued at 6-8 minutes when the licensing reaction was fully activated. In samples supplemented with D-box no activity was observed at early timepoints before a switch like activation in licensing activity was observed at 6-8 minutes (Figure 22). This suggests that the activation of replication licensing requires at least 6-8 minutes of ongoing APC/C activity, after which point the APC/C is no longer required.

In the D-box assay, plotting the control samples at the same timepoints as the D-box samples can be considered somewhat misleading (Figure 22). In reality, these samples

are not inhibited by the addition of buffer and therefore these samples have an extra 6 minutes to activate the licensing machinery and carry out replication licensing before

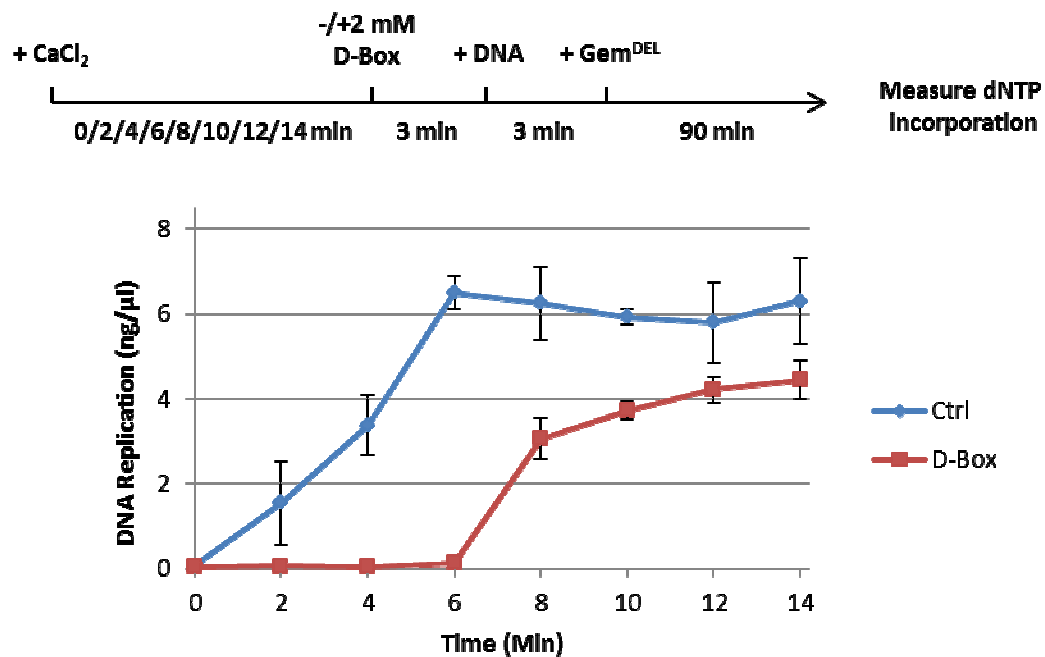


Figure 22: The APC/C Regulates a Switch in Licensing Activity after 6-8 Minutes. Extracts were supplemented with Buffer (LFB1/50) or 2 mM D-box peptide at defined times post calcium addition. DNA was added 3 minutes later and incubated for 3 minutes to allow for minimal licensing. Licensing was stopped by addition of 100 nM His₆-Geminin^{DEL}. Reactions were incubated for a further 90 minutes before DNA replication was measured. Average of 3 experiments; error bars represent standard error.

the addition of geminin^{DEL} compared to the treated samples. For a more appropriate control the D-box assay was repeated with DNA addition following 1 minute after D-box addition (Figure 23). These experiments were also performed in a different extract, which had slightly slower kinetics. In the control sample a linear increase was observed which began to plateau after 10 minutes. D-box treated samples again demonstrated an APC/C-dependent switch in the activation of licensing, this time occurring at 8-10 minutes (Figure 23A). Reducing the incubation time of the D-box prior to DNA addition was successful in decreasing the gap between the control and treated samples.

This switch-like activation of licensing could be due to the regulation of CDK activity, geminin activity or both simultaneously. It is difficult to determine whether this effect is

due to geminin inactivation directly, however, the role of CDK activity can be tested using a CDK inhibitor. To determine which of these two APC/C targets is responsible for the switch in activity, the D-box assay was repeated with the addition of 6-DMAP at the time of D-box addition to inhibit CDK activity (Figure 23B). 6-DMAP was used as it was previously shown to inhibit mitotic CDK activity with only small effects on interphase CDK activity required for the initiation of replication (Blow, 1993). If APC/C dependent inactivation of CDK was responsible for the switch in licensing activation, then D-box/6-DMAP samples should closely resemble the control experiment. Whereas the addition of 6-DMAP did result in a faster rise in licensing activity in the control sample and the D-box samples, it did not alter the observed switch in activation (Figure 23B). Even in the presence of 6-DMAP, early timepoints do not contain any licensing activity, differing substantially from the control samples. This demonstrates that the target downstream of APC/C responsible for the switch in licensing activity is not CDKs. Therefore, geminin inactivation is likely to be responsible for the observed kinetics.

APC/C-dependent ubiquitination typically results in destruction of target proteins by the 26S proteasome. To gain more information about the nature of the switch in licensing activity the D-box assay was carried out in the presence of 0.1 mM bortezomib to inhibit the proteasome (Figure 23C). The addition of bortezomib would determine if the switch required APC/C mediated protein destruction, or an alternative outcome of APC/C activity. Inhibition of the proteasome had no effect on the appearance of the switch, suggesting that protein destruction is not required. This is consistent with data that shows the proteasome is not required for licensing or DNA replication (Li and Blow, 2004). Therefore an APC/C dependent event which does not induce protein destruction is responsible for a switch like activation of licensing which is most likely acting through geminin.

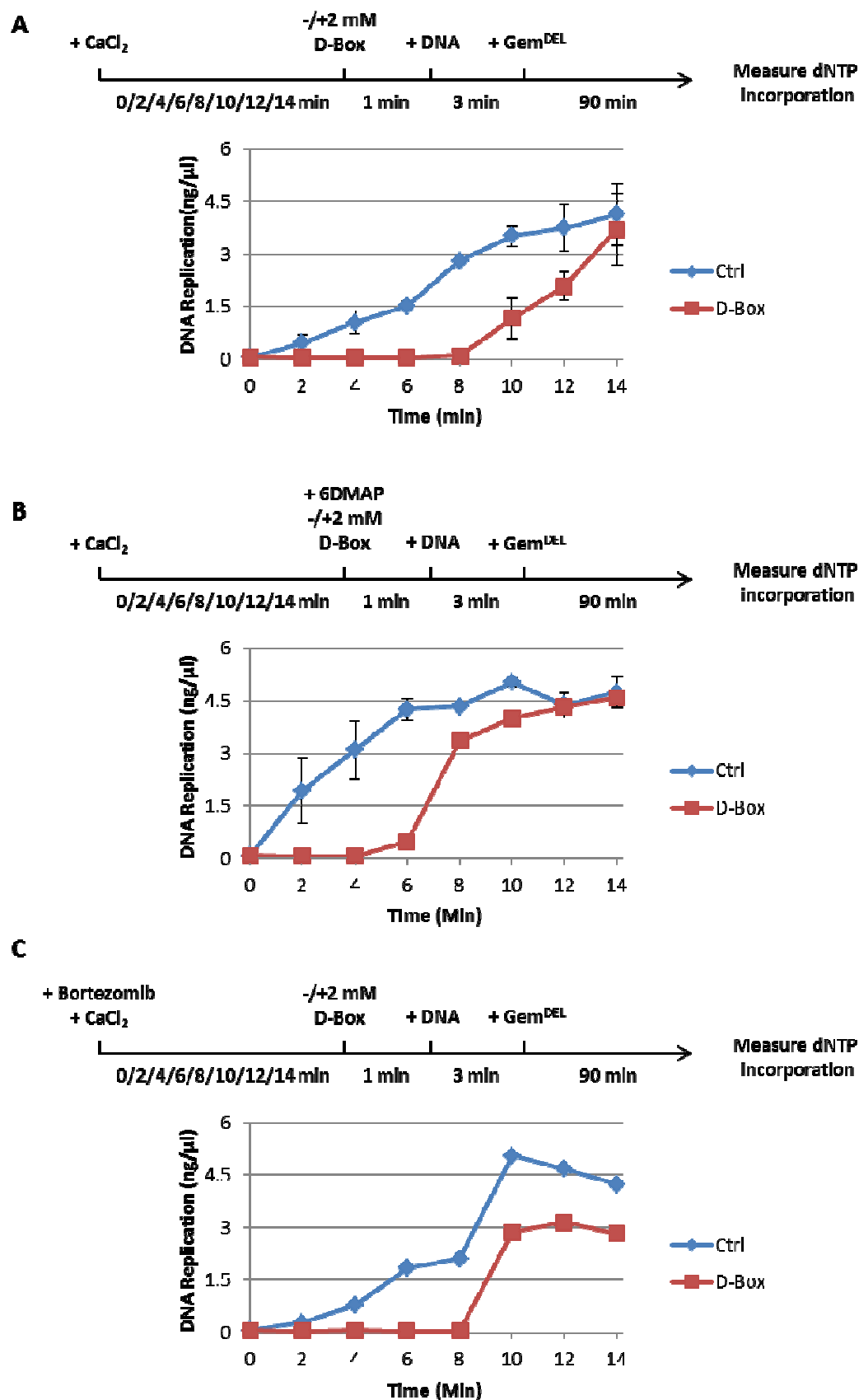


Figure 23: APC/C-dependent Switch is not due to Cyclin B Regulation and does not Require Protein Degradation. (A) Extracts were supplemented with Buffer (LFB1/50) or 2 mM D-box peptide at

defined times after calcium addition. DNA was added 1 minute later and incubated for 3 minutes to allow for minimal licensing. Licensing was stopped by addition of 100 nM His₆-Geminin^{DEL} and reactions were incubated for a further 90 minutes before DNA replication was measured. Average of 3 experiments; error bars represent standard deviation. (B) Reactions were carried out as in (A), with 3 mM 6-DMAP addition in all samples at defined times supplemented together with either Buffer (LFB1/50) or D-box. (C) Metaphase extracts were pretreated with 0.1 mM bortezomib before reactions were carried out in (A).

The control samples in the D-box experiments do not truly represent the licensing activity at the timepoints shown (the time when D-box peptide was added) as the licensing activity can continue to increase throughout the experiment until geminin^{DEL} is added, giving these samples an additional 4-6 minutes compared to D-box samples. To define the times more precisely an additional control was performed where DNA was added to extract at different times, followed 3 minutes later by geminin^{DEL} addition (Figure 24). Licensing activity showed a linear increase and reached its maximal value at 8-10 minutes after calcium addition, in line with previous experiments. These results confirm the idea that APC/C activity is required for 8-10 minutes for geminin to become stably inactivated, and that continued APC/C activity is not required for the inactive state of geminin to be maintained.

4.3 The Ubiquitination of Geminin

We have found that there is an APC/C-dependent switch in the ability to license DNA 8-10 minutes after calcium addition. This does not result from CDK inactivation, suggesting that this effect is mediated downstream of APC/C leading to the inactivation of geminin (Figure 23B). This could be due to direct APC/C mediated ubiquitination of geminin or indirect due to APC/C acting on another unidentified protein (i.e. APC/C is upstream of geminin in a pathway that results in geminin inactivation). It was also shown that this switch does not require protein destruction. Li and Blow proposed that geminin is ubiquitinated prior to inactivation (Li and Blow, 2004). This predicts that all geminin is subjected to ubiquitination, with some, but importantly not all, being destroyed with some being deubiquitinated and subsequently inactivated. One caveat to

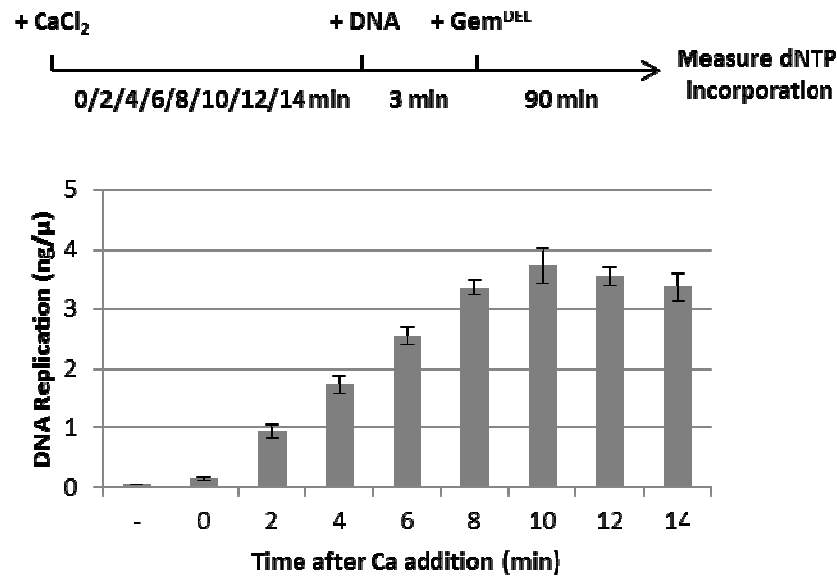


Figure 24: Licensing Activity Occurs within 8 minutes of Calcium Addition. DNA was added to extracts at defined times after calcium addition and incubated for 3 minutes before supplementation with 100 nM His₆-Geminin^{DEL}. DNA replication was measured after a further 90 minutes.

this conclusion is that experiments showing ubiquitinated species of geminin focused on the ubiquitination of recombinant geminin rather than endogenous protein, which is preferentially degraded rather than inactivated in extracts (Hodgson et al., 2002, McGarry and Kirschner, 1998). Furthermore, the quantity of endogenous geminin that is subjected to ubiquitination was not investigated, therefore it is possible that only the portion of geminin that is targeted for destruction is ubiquitinated while the portion that becomes inactivated may be regulated by an alternative method that requires APC/C activity upstream. Addition of K48R ubiquitin (Lysine 48 mutated to Arginine) was shown to stabilise geminin and prevent changes in geminin-Cdt1 complex formation, suggesting that K48 linked chains were required for geminin inactivation (Li and Blow, 2004). However, the K48R mutant could have prevented inactivation of Emi2 to block exit from mitosis. Additional controls would have clarified whether extracts in this experiment had failed to inactivate geminin specifically or had failed to exit mitosis, as it is difficult to study these as separate APC/C-dependent processes.

We attempted to determine whether direct ubiquitination of geminin was a prerequisite for geminin inactivation (Figure 25A). To undertake these studies ubiquitin vinyl sulfone (UbVS) was used to inhibit deubiquitinases (DUBs). Metaphase or interphase extract was untreated or supplemented with 20 μ M UbVS for 30 minutes. This results in a complete inhibition of DUBs and subsequently free ubiquitin is depleted from the extract as it is sequestered in large conjugates generated by the proteasome, preventing ubiquitin turnover. The depletion of large ubiquitin ubiquitin conjugates serves as a positive control for the activity of UbVS as a DUB inhibitor. UbVS treated/ubiquitin depleted extract was supplemented with 44 μ M wild-type (wt) ubiquitin and reformation of high molecular weight complexes was observed at 0, 25, 45 and 75 minutes after addition of wt-ubiquitin. This experiment demonstrates the effectiveness of UbVS as a DUB inhibitor and shows that ubiquitin depletion can be rescued by the addition of exogenous protein.

To determine whether protein ubiquitination in general is required for replication licensing metaphase extracts were depleted of ubiquitin by the addition of UbVS for 30 minutes (Figure 25B). This resulted in a failure to activate licensing in response to calcium addition. However, licensing was rescued by the addition of exogenous ubiquitin. This demonstrates that ubiquitin is required for the activation of licensing, but DUBs are dispensable. To demonstrate that the loss of MCM loading upon UbVS addition was due to a failure to activate the licensing system, rather than being a specific some other step of MCM loading, the previous experiment was repeated in interphase extract. Extracts were released into interphase before UbVS treatment and the depletion of ubiquitin (Figure 25C). When the licensing machinery was previously activated by the addition of calcium, the rescue of ubiquitin depletion with exogenous protein was not required for licensing. This demonstrates that ubiquitination, but not DUB activity, is required specifically for the activation of replication licensing.

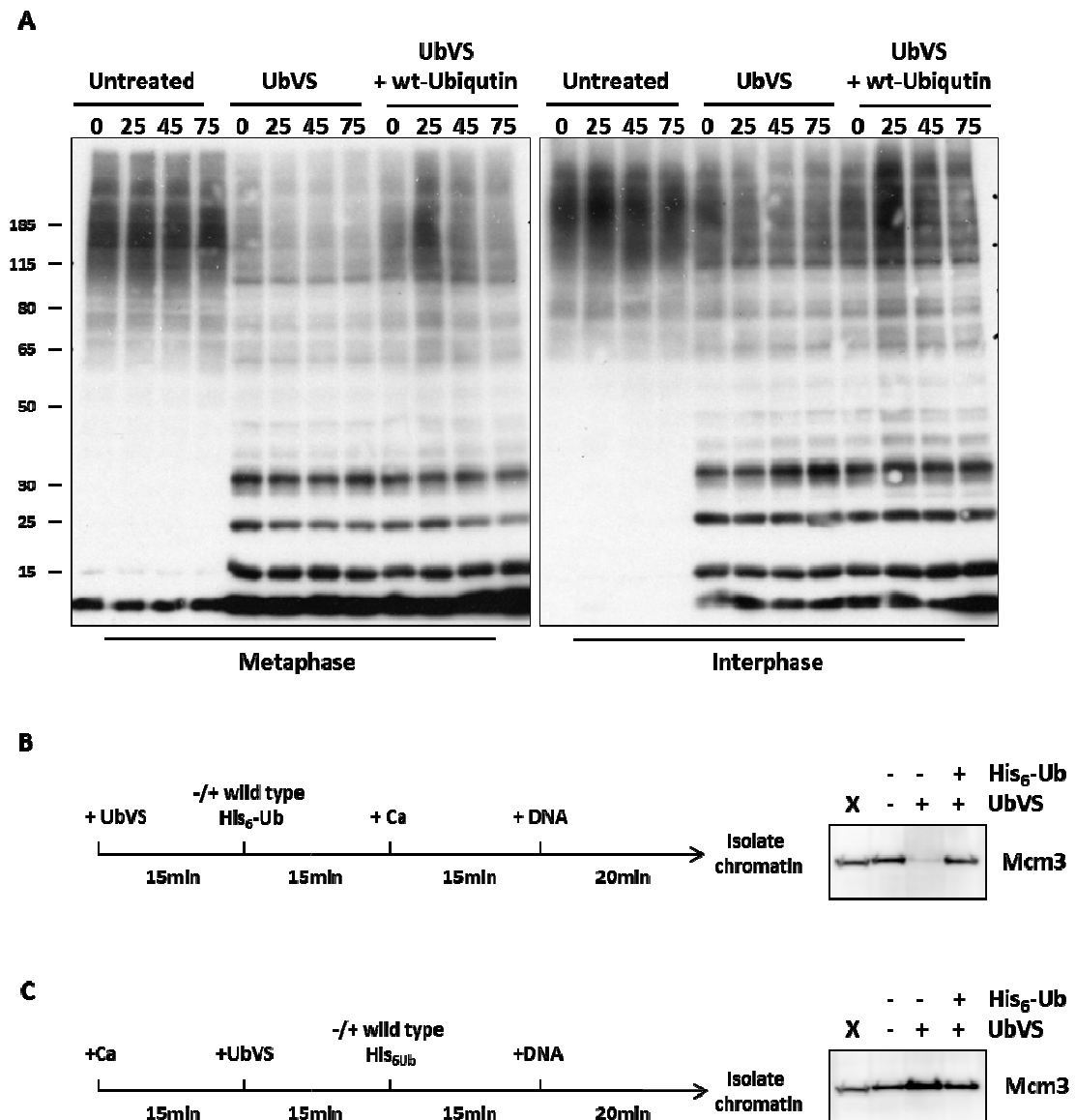


Figure 25: UbVS inhibits Deubiquitinases Resulting in Ubiquitin Depletion. (A) Metaphase or interphase extract was untreated or supplemented with 20 μ M UbVS for 30 minutes. UbVS treated extract was subsequently untreated or supplemented with 44 μ M wild-type (wt) ubiquitin. Samples were taken at 0, 25, 45 and 75 minutes after ubiquitin addition and analysed by western blot against ubiquitin to assess reformation of high molecular weight ubiquitin complexes. (B) Untreated, UbVS treated/ubiquitin depleted, and UbVS treated/ubiquitin rescued metaphase extract was supplemented with calcium and incubated for 15 minutes. DNA was added and incubated for 20 minutes before chromatin was isolated and analysed by western blot to determine the levels of licensing. (C) Untreated, UbVS treated/ubiquitin depleted or UbVS treated/ubiquitin rescued interphase extract was supplemented with DNA and incubated for 20 minutes. Chromatin was isolated and analysed by western blot to determine the levels of licensing.

The activation of replication licensing requires APC/C activity and free ubiquitin. The UbVS reagent can be applied to gather more information about the type of ubiquitination that is being undertaken in extracts. Metaphase extracts were depleted of ubiquitin by UbVS treatment. To determine what kind of chain types are required for the activation of licensing, depleted extracts were rescued with various ubiquitin mutants (Figure 26B). The APC/C catalyses mainly the addition of K11 chains on substrates and some K48 linked chains. Metaphase extracts were then supplemented with methylated ubiquitin (which cannot form chains), K48R ubiquitin (which cannot form K48 linked chains), K48O ubiquitin (which can only form K48 chains), K11R ubiquitin (which cannot form K11 linked chains) and K11O ubiquitin (which can only form K11 linked chains). Extracts were released into interphase by calcium addition and the level of licensing activity determined by chromatin isolation and western blot of Mcm2 and Mcm7. There was a small level of background licensing in the ubiquitin depleted control, however addition of wild-type ubiquitin resulted in a strong activation of licensing. Equal levels of activity were observed for K48R ubiquitin, demonstrating that K48 chains are dispensable for replication licensing. Methylated ubiquitin, K48O and K11O all resulted in a reduced level of replication licensing suggesting that mono-ubiquitination, multiple mono-ubiquitination, K48 chains and K11 chains are all able to influence replication licensing activation to some extent. K11R ubiquitin strongly inhibited licensing suggesting that K11 chains are essential for the activation of licensing.

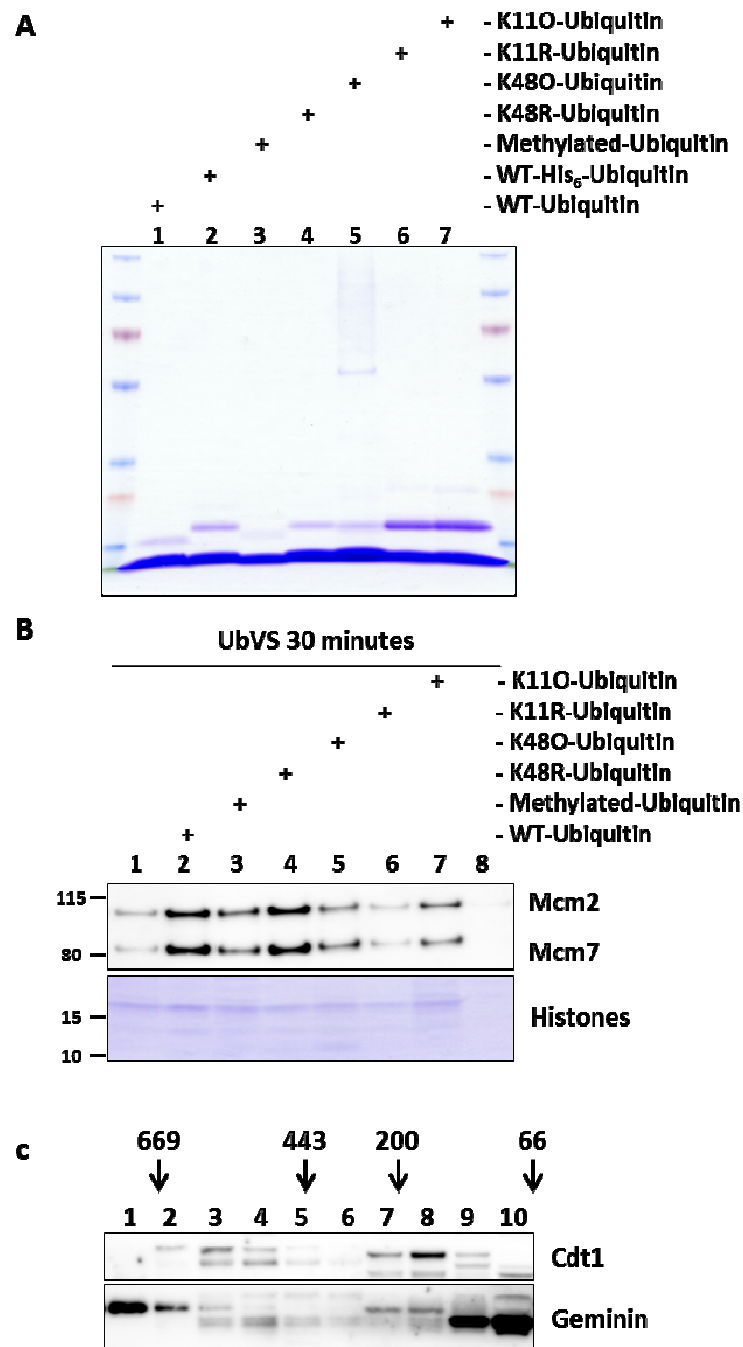


Figure 26: Chain Types Required for Licensing and the Reactivation of Geminin. (A) Ubiquitin reagents were separated on an SDS-PAGE gel and stained with coomassie to ensure the concentrations were relatively similar. (B) Metaphase arrested extract was supplemented with UbVS and incubated for 30 minutes to deplete ubiquitin. Extracts were supplemented with different ubiquitin mutants prior to the addition of 10 ng/μl DNA. Chromatin was isolated after 20 minutes and the levels of licensing were determined by western blot for Mcm2 and Mcm7. A coomassie stain of histones is shown as a loading control. (C) Interphase extract was supplemented with UbVS and ubiquitin to inhibit DUBs and prevent ubiquitin depletion. DNA was added at 10 ng/μl and extracts were incubated for 90 minutes before being diluted 1/4 with LFB2/50 containing 0.1% Triton to lyse nuclei and centrifuged to remove DNA. The supernatant was filtered through a 0.2μm membrane and fractionated by size exclusion chromatography. Fractions were analysed by western blot for geminin and Cdt1.

As a simple control during this experiment to determine whether K11R ubiquitin addition inhibited replication licensing activation specifically or prevented the exit from metaphase arrest, nuclear formation was analysed by UV/phase contract microscopy. Interestingly, all UbVS-treated samples, including wild type rescues, were unable to form nuclei or decondense sperm DNA. Therefore this assay could not be used to determine the specificity of K11R's effect on replication licensing. The inability to form nuclei was tested further and it was found that addition of UbVS to interphase extract before DNA addition did not affect nuclear formation, suggesting DUBs are required to facilitate nuclear formation and chromatin decondensation specifically upon exit from metaphase arrest.

Li and Blow hypothesised that geminin ubiquitination is a prerequisite to geminin inactivation. To learn more about the role of geminin ubiquitination, treating extracts with UbVS, bortezomib and rescuing ubiquitin depletion with tagged forms of ubiquitin would allow for the identification of ubiquitinated geminin species by pulldowns. These kinds of experiments would give information about the chain types and kinetics of geminin ubiquitination, but would not give any information about the activity of geminin. If the hypothesis of Li and Blow is correct, the bulk of geminin is ubiquitinated, resulting in the degradation of some, but the remainder is subjected to DUB activity as a prerequisite to inactivation. If this is true, the addition of UbVS should block the reactivation of geminin. Geminin becomes reactivated after nuclear import and complex formation with Cdt1 can be seen by size exclusion chromatography. An experiment was undertaken to determine if UbVS blocks the reactivation of geminin in pre-incubated extracts (Figure 26C). Interphase extract was treated with UbVS for 15 minutes to ensure DUB inhibition and supplemented with wild type ubiquitin to prevent depletion. DNA was added at 10 ng/ μ l and extracts were incubated for 90 minutes before the generation of a soluble 'pre-incubated extract' (PIE)

by dilution and centrifugation. The pre-incubated extract was then fractionated by size exclusion chromatography and fractions were analysed by western blot for geminin and Cdt1 complex formation. A geminin-Cdt1 complex between 669 and 443 kDa had reformed (Figure 26C). Nuclear formation was observed by UV/phase microscopy at 90 minutes to ensure extracts had progressed through the cell cycle. Large round nuclei with recondensed chromatin were observed indicating that extracts were in interphase and the complex observed on gel filtration could not simply be a metaphase arrested complex. This experiment shows that geminin deubiquitination is not required for its reactivation. It also shows that not all geminin is subjected to ubiquitination as this experiment was carried out in the absence of DUBs but in the presence of the 26S proteasome, therefore all ubiquitinated species were presumably destroyed.

4.4 Investigating the Role of Geminin Phosphorylation

It has been reported that geminin is modified by multiple PTMs in human, mice and rat cells (Figure 27). The majority of these modifications are phosphorylation. Whereas the majority of these reports are from big data experiments that search for specific PTMs, a number of reports have investigated the role of geminin modifications directly (Roukos et al., 2007, Zhou et al., 2012, Tsunematsu et al., 2013, Blanchard et al., 2014). Attempts have been made in the Blow laboratory to investigate geminin phosphorylation in the context of geminin reactivation. Incubation of interphase geminin separated by gel filtration or immunoprecipitated interphase geminin with lambda phosphatase did not increase the interaction of interphase geminin with His₆-Cdt1 (Hodgson, B., unpublished observation). This data did not support a role for dephosphorylation as a geminin activator. Previous attempts have been made in the Blow laboratory to identify novel geminin PTMs by mass spectrometry analysis of geminin immunoprecipitated from metaphase and interphase *Xenopus* egg extracts (Gillespie, P.J., DiSanto, R., unpublished observation). These previous efforts did not

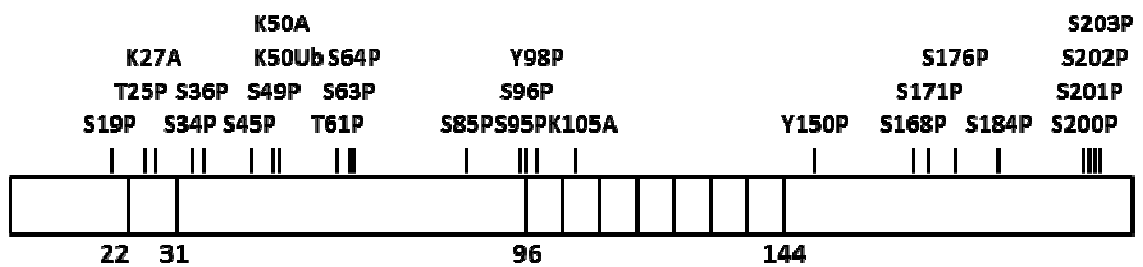


Figure 27: Multiple Modifications of Human Geminin. A complete list of geminin modifications that have been identified by mass spectrometry is available at www.phosphosite.org. Human geminin is shown with the residue position and type of modification identified. Some modifications were identified in mice or rats and are shown on the equivalent residue in human protein. Multiple modifications have been verified by methods other than mass spectrometry. Modifications are clustered in the N-terminus near the motifs that regulate geminin stability. The D-box motif is shown from 22-31. A second cluster of modifications is located near the coiled-coil, which is shown from 96-144. The coiled-coil is responsible for geminin dimerization and Cdt1 binding. A third cluster in the C-terminus has been shown to regulate geminin cleavage after caspase activation. P- phosphorylation, A- acetylation Ub- ubiquitination.

identify novel *Xenopus* geminin modifications but was also unable to conclusively rule out PTM as a method for geminin regulation and therefore this mechanism warranted further investigation.

To gain evidence supporting a role for phosphorylation in the regulation of licensing, experiments were designed to inhibit kinases and phosphatases known to function at relevant stages of the cell cycle and determine the effects on DNA replication licensing. Metaphase or interphase extract was supplemented with inhibitors and chromatin was isolated and analysed by western blot to determine the effect of drug addition on Mcm3 chromatin loading(Figure 28).Plk1 inhibition by BI2456 and inhibition of the APC/C by a D-box peptide had a significant effect resulting in the inhibition of licensing when added to metaphase extract. From this type of experiment, however, it was not possible to determine whether this effect was due to a direct interaction with the licensing reaction, or an indirect inhibition of licensing due to issues with mitotic exit.

To look directly for novel phosphorylations and other PTMs of *Xenopus* geminin, geminin immunoprecipitated from metaphase and interphase extracts were trypsinised

Metaphase

GemininH 123/219 (56.2% Coverage)

MNTNKKQRLD MEKPTMSIKN YFVDKTNESL APRRTLKVIQ
PSASGCLVGR TKEPVKNSTK RKLWNDQLTS KKAKVEVAVD
PEHRENKDCS SEAYDLMVKE TPTCLYWKEV AEERRKALYE
ALQENEKLHK EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PRSLEDLKD DLEEARFEDE ADMAEARIED
ETDMARPSNS DQNMDAHTV

Geminin L 109/216 (50.5% Coverage)

MNSNMKQSRD VENPSMSIKN YIVDKTNEAL APRRTLKVIQ
QSASGCLVGR TKEPAKNSTK RKLWNDQLTS KKAKVEVAVD
PEHQENKDCP SEAYDLMVKE TPTCLYWKDV AEERRKALYE
ALQENEKLHQ EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PQSLEDLKNL DLEEARFEDE AESRIEDET
MTQPSSSDQN MDKQTV

Interphase

GemininH 123/219 (56.2% Coverage)

MNTNKKQRLD MEKPTMSIKN YFVDKTNESL APRRTLKVIQ
PSASGCLVGR TKEPVKNSTK RKLWNDQLTS KKAKVEVAVD
PEHRENKDCS SEAYDLMVKE TPTCLYWKEV AEERRKALYE
ALQENEKLHK EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PRSLEDLKD DLEEARFEDE ADMAEARIED
ETDMARPSNS DQNMDAHTV

Geminin L 101/216 (46.8% Coverage)

MNSNMKQSRD VENPSMSIKN YIVDKTNEAL APRRTLKVIQ
QSASGCLVGR TKEPAKNSTK RKLWNDQLTS KKAKVEVAVD
PEHQENKDCP SEAYDLMVKE TPTCLYWKDV AEERRKALYE
ALQENEKLHQ EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PQSLEDLKNL DLEEARFEDE AESRIEDET
MTQPSSSDQN MDKQTV

Figure 29: Coverage of Geminin Peptides. The combined coverage of peptides from trypsinised and phosphoenriched geminin immunoprecipitation samples is shown, highlighted in blue. Motifs that regulate protein stability, mediate Cdt1 interaction and make up the coiled-coil are underlined. There was moderate coverage of geminin H and geminin L in both metaphase and interphase. However, no modified peptides were identified.

4.5 Reactivation of Geminin Over Time

The identification of an on/off switch mechanism fits well with the hypothesis that geminin is regulated by post-translational modification. It is likely that a portion of geminin is targeted for degradation and the remainder is regulated by a mechanism

other than ubiquitination. Initial mass spectrometry experiments were unsuccessful in identifying geminin PTMs, therefore an alternative method was required to find evidence supporting the existence of a modification. 2D gel electrophoresis separates proteins by two intrinsic properties. First proteins are separated by charge on an isoelectric focusing (IEF) strip and second by size on a SDS-PAGE gel. Specific spots are then identified by western blot.

1 µl equivalents of metaphase and interphase extracts were separated on 7 cm pH 3-5.6 and pH 3-11 IEF strips (Figure 30). The theoretical pI of geminin H and L is 5.06 and 4.81, respectively. Therefore two spots are expected for geminin. 2D gels produced variable results, but overall the data suggested that multiple charged forms of geminin exist which vary depending on the cell cycle stage. This difference was more apparent on the pH 3-11 strip (Figure 30B). It is tempting to speculate that these charge states could be due to PTMs during the cell cycle.

If there is a modification, there must be an explanation as to why it has not been identified by mass spectrometry. This has been attempted previously without success in the Blow laboratory. One simple explanation is that peptides which are consistently not identified could contain modifications. Increasing the input of immunoprecipitated geminin and analysing duplicate samples with a combination of proteases should increase coverage and the likelihood of identifying any potential modifications. A second explanation could be that the modification is lost during the immunoprecipitation. There is not sufficient endogenous geminin to analyse whole extracts directly, therefore immunoprecipitations must be performed. However, protocols that require long incubation times could allow for a slow loss of modifications over time. It has been reported that the ability to license chromatin is lost over time in *Xenopus* interphase extracts (Blow, 1993, Mahbubani et al., 1997). This was thought to

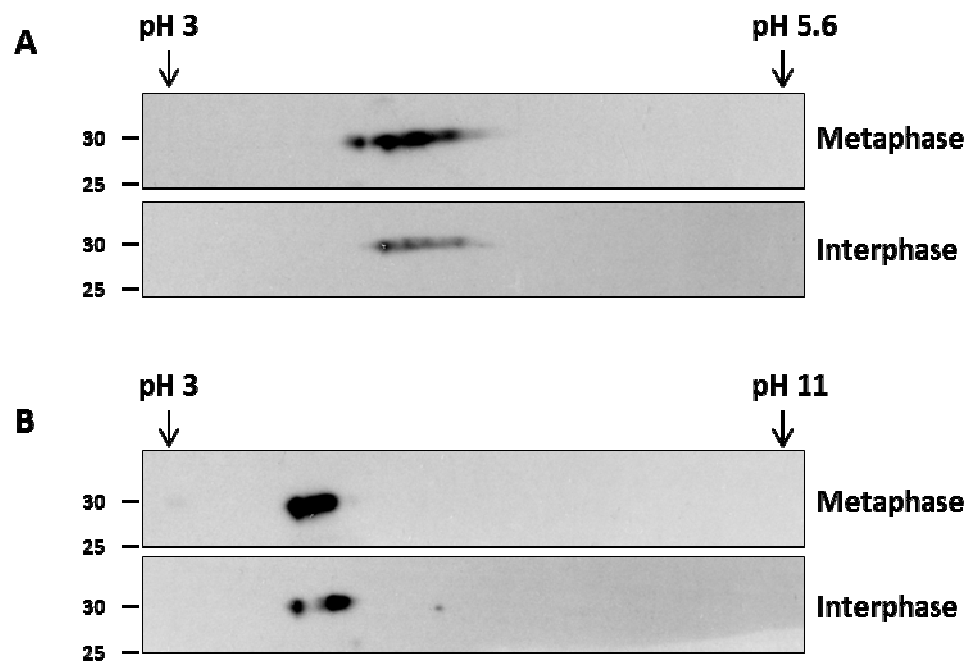


Figure 30: Multiple Forms of Geminin Identified by 2D Gel Electrophoresis. (A) Metaphase and interphase extracts were denatured in rehydration buffer and 1 μ l equivalents were used to rehydrate pH 3-5.6 IEF strips overnight. Proteins were separated by isoelectric focusing for a total of 33,000 voltage hours followed by SDS-PAGE and western blotting for geminin. (B) The experiment was carried out as in (A) on a pH 3-11 IEF strip.

be due to a loss of Cdt1 activity between 60-120 minutes after calcium addition (Mahbubani et al., 1997). It was later shown that Cdt1 is targeted for destruction by the APC/C (Li and Blow, 2005) and it was hypothesised in our laboratory that ongoing APC/C-dependent destruction of Cdt1 may account for this loss of activity. However, it was demonstrated that Cdt1 levels remain above ~50% for at least 90 minutes after entry into interphase (Figure 11). An alternative explanation for this loss of Cdt1 activity could be a reactivation of geminin over time in extract. Therefore it was attractive to investigate whether this loss in Cdt1 activity was due to a reactivation of geminin over time rather than destruction of Cdt1.

To test geminin activity over time the most appropriate assay was to observe complex formation with Cdt1. Extract was fractionated by size exclusion chromatography at various times after calcium addition and fractions were analysed by western blot for

geminin and Cdt1 (Figure 31A). Cdt1 and geminin eluted in the expected fractions in metaphase and 15 minute interphase extracts (compared to Figure 13). After further incubation the geminin complexes shifted into higher molecular weight complexes concurrently with Cdt1. Western blotting during these experiments produced excessive background as non-specific bands become more intense over time. This experiment was carried out multiple times with multiple extracts, each yielding reformation of geminin-Cdt1 complexes. To further demonstrate the reformation of high molecular weight complexes geminin bands in lanes 1-8 were quantified by 2D densitometry and plotted as a percentage of total protein per lane over time (Figure 31B). This suggests that geminin may reform a high molecular weight complex over time in interphase.

4.6 Identification of Novel Geminin Post-Translational Modifications

Previous experiments undertaken by the Blow laboratory which aimed to identify novel geminin PTMs by mass spectrometry were unsuccessful. Early work carried out during this project also failed to identify modifications by mass spectrometry. Geminin reactivation was subsequently observed over time in interphase extracts, therefore the likelihood that previous mass spectrometry experiments provided false negatives increased, as geminin samples may have been reactivated during the long incubation times of the IP protocols. To get the most reliable and conclusive data on geminin PTMs it was critical that geminin IPs were optimised to ensure extracts still maintained inactive geminin. Geminin appears to reactivate as soon as 30 minutes after calcium addition (Figure 31), therefore an optimal protocol is required to complete geminin IPs within 30 minutes of calcium addition.

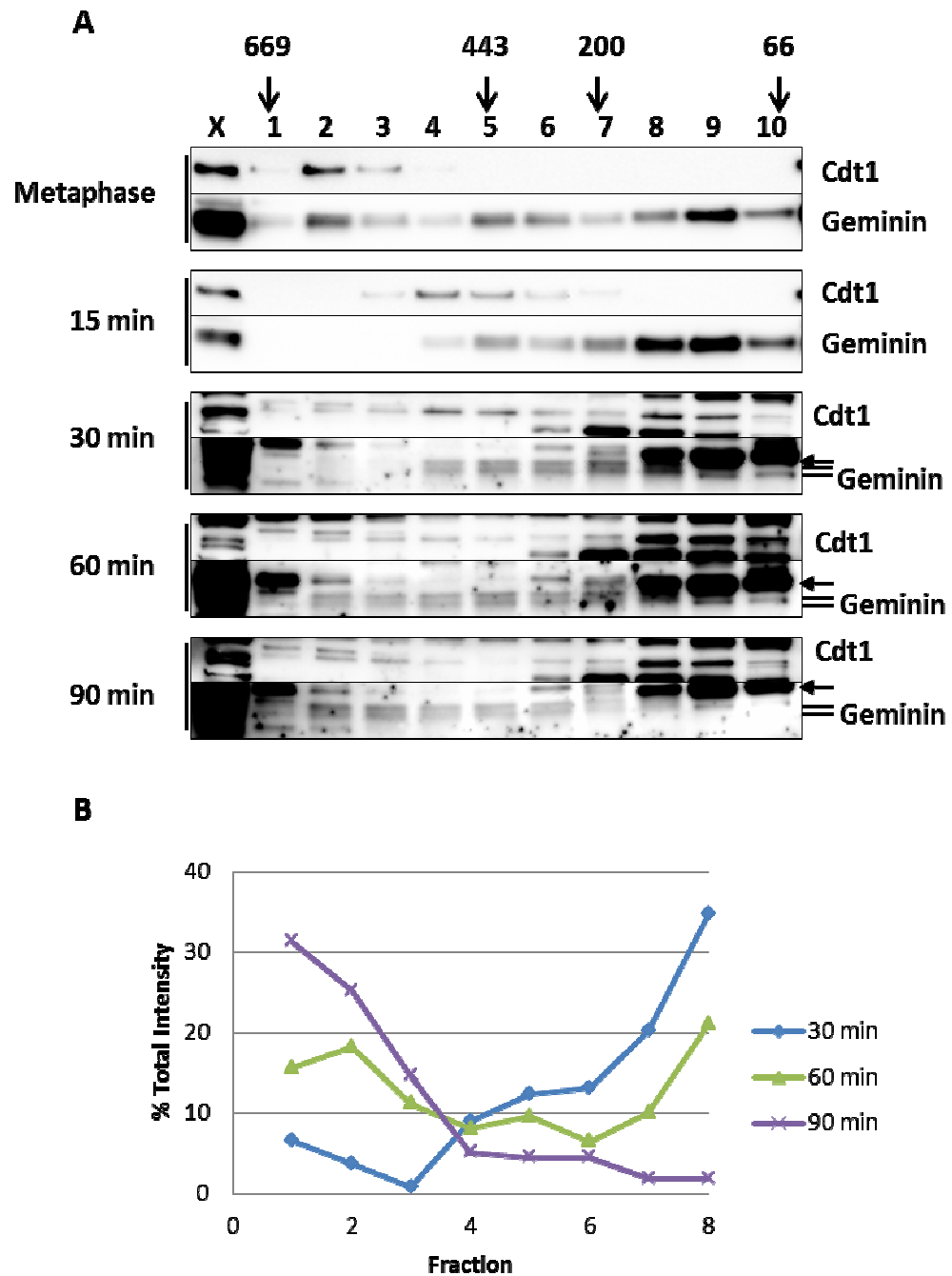


Figure 31: Geminin Reactivates Over Time. (A) Metaphase extract was released by calcium addition and extracts were incubated at 20°C for various times before being diluted 1/5 with LFB1/50, spin filtered through a 2 µm membrane and applied to a MAbPAC SEC-1 column for size exclusion chromatography. Metaphase extract was fractionated to show the high molecular weight complex of active geminin bound to Cdt1. X denotes 0.5 µl equivalents of input samples. Arrow indicates non-specific band. Double line indicates geminin. Metaphase and 15 minute samples were not run long enough on SDS-PAGE to separate the non-specific band from geminin in lanes 8, 9 and 10. (B) The geminin bands in lanes 1-8 were quantified by 2D densitometry. Samples are plotted as a percentage of total intensity to demonstrate the movement of geminin from right to left over time, consistent with complex reformation.

A geminin IP protocol using crosslinked protein-A Dynabeads was optimised to determine an incubation time and dilution factor that allows a pulldown of geminin to near depletion levels, as quickly as possible (see methods 2.5.7). It was important to include a dilution to quickly cool extracts to 4°C to slow down enzyme activities. Increasing the dilution factor reduced the speed of the pulldown (compare lanes 2 and 3) and increasing the time resulted in a greater pulldown (compare lane 3 and 7). A dilution factor of 1/2 and an incubation of 10 minutes resulted in near depletion levels of geminin and complete depletion of Cdt1 in metaphase extract. The level of depletion was not increased with a longer incubation time (Figure 32A: compare geminin levels in lane 10 and 12). Therefore this was selected as the optimal condition. Two bands remain in the supernatant and it is likely that the stronger band is the same non-specific band observed close to geminin on size exclusion chromatography (Figure 14). Interphase extracts were also tested for all optimisation conditions as there is a much greater viscosity in these extracts, which could have required an alternative protocol for fast efficient IPs. However, a 1/2 dilution for 10 minutes also provided the most efficient IP in interphase extracts (Figure 32). This pulled down the greatest amount of geminin with the least amount of Cdt1 co-IP, suggesting it contains mostly inactive geminin (Compare lanes 6 and 8).

The optimised geminin immunoprecipitation protocol could now be used to provide samples for mass spectrometry. This protocol gives some assurance that metaphase and interphase samples contain a majority of active and inactive geminin protein, respectively. From the time of calcium addition until the final wash of the IP and denaturation of samples by boiling in gel loading buffer, no longer than 30 minutes will have passed; at this timepoint the majority of geminin runs at a low molecular weight on gel filtration indicative of inactive protein (Figure 31). Having this confidence in the activity of the sample is essential to form a conclusion on the existence of

Xenopus geminin PTMs, either positively or negatively, assuming a good coverage of proteins is obtained.

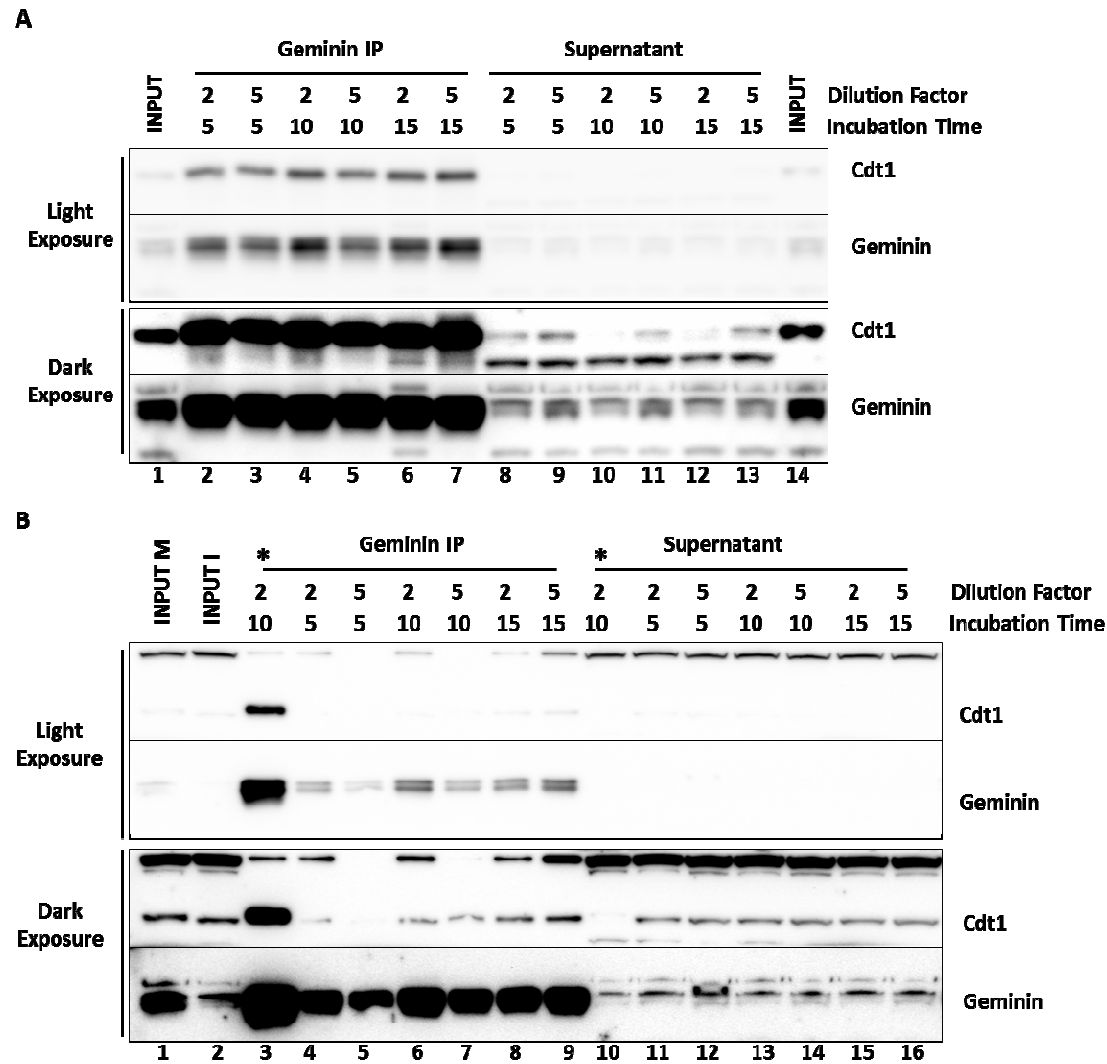


Figure 32: Optimisation of Geminin Immunoprecipitation. (A) Metaphase extracts were mixed 1:1 with protein A DynaBeads crosslinked with geminin antibody serum, diluted 1/2 or 1/5 with ice cold LFB1/50 and incubated for 5, 10 or 15 minutes. Beads were isolated and washed thoroughly. 5% of input, an equivalent amount of bead flowthrough and the immunoprecipitated samples were analysed by western blot to determine the levels of geminin pulldown and depletion. Two exposures are shown to demonstrate IP samples and supernatants at relevant levels. (B) Experiments were carried out as in (A) with interphase extracts. A metaphase immunoprecipitation was run as a positive control represented by a star.

Duplicate geminin 10 minute IP samples were prepared from metaphase and interphase extracts. To ensure a more complete coverage of regions which are consistently and repeatedly unidentified, duplicate samples were prepared for digestion with trypsin and Lys-C. A control western blot was performed to show the difference in activity between

metaphase and interphase samples (Figure 33A). The reduction of geminin levels and of Cdt1 co-IP suggest that activities are maintained during the IP. Samples were resolved by SDS-PAGE and stained with Coomassie (Figure 33B). In the event that modified geminin proteins are less efficient antigens the input and flowthrough samples were also included for mass spectrometry analysis. Supernatant samples were slightly overloaded compared to inputs. Specific bands were excised and digested with trypsin or Lys-C and peptides were analysed by MS/MS. A recombinant full length geminin sample was also included as a technical control.

The coverage of geminin H and L proteins from each sample is summarised in Table 3. A visual representation of peptide coverage of geminin H and L proteins is also shown (Figure 34, Figure 35 and Figure 36). An almost complete coverage of recombinant geminin H peptides was obtained (97.7% coverage with trypsin and Lys-C combined) showing that samples were efficiently digested and that good coverage of IP samples should be expected. A low coverage of peptides was obtained in metaphase input and flowthrough samples (Figure 34). However, both interphase input and flowthrough samples had an increased coverage compared to metaphase equivalents (Figure 35). The interphase flowthrough sample gave an unexpectedly high coverage of 58% for geminin H suggesting that the IP was less efficient compared to metaphase samples, however there was some overloading observed on the input gel which may account for additional protein. Metaphase IP samples gave a good coverage of 85% and 88% for geminin H and L respectively. Identification of interphase peptides was slightly lower with 74% and 77% coverage of Geminin H and L respectively. These experiments resulted in the identification of significantly more geminin peptides, giving a more complete coverage, compared to previous experiments. The loss of some peptides in interphase compared to metaphase IPs could be due to the quantity of input, as less geminin is present in interphase extracts. Alternatively, interphase geminin may

be a less efficient antigen due to modification, which is consistent with the high coverage obtained from interphase flowthrough samples.

Geminin	Enzyme	Meta			Inter			Recombinant
		input	IP	FT	Input	IP	FT	
H	Trypsin	0	76.70%	0	10%	69%	55%	95.89%
	Lys-C	5.47%	47%	19.17%	10.90%	42.90%	5.40%	63%
	Combined	5.47%	85.85%	19.17%	21%	74.43%	58.45%	97.72%
L	Trypsin	6.01%	84.9%	15.27%	25%	65.74%	11.50%	-
	Lys-C	0	65.27%	0	0	62.50%	0	-
	Combined	6.01%	88.88%	15.27%	25%	77.31%	11.57%	-

Table 3: Summary of Geminin Coverage by Mass Spectrometry

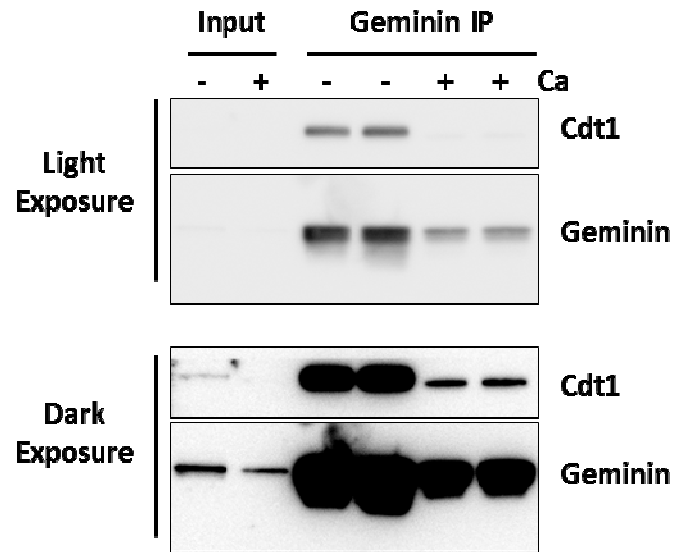
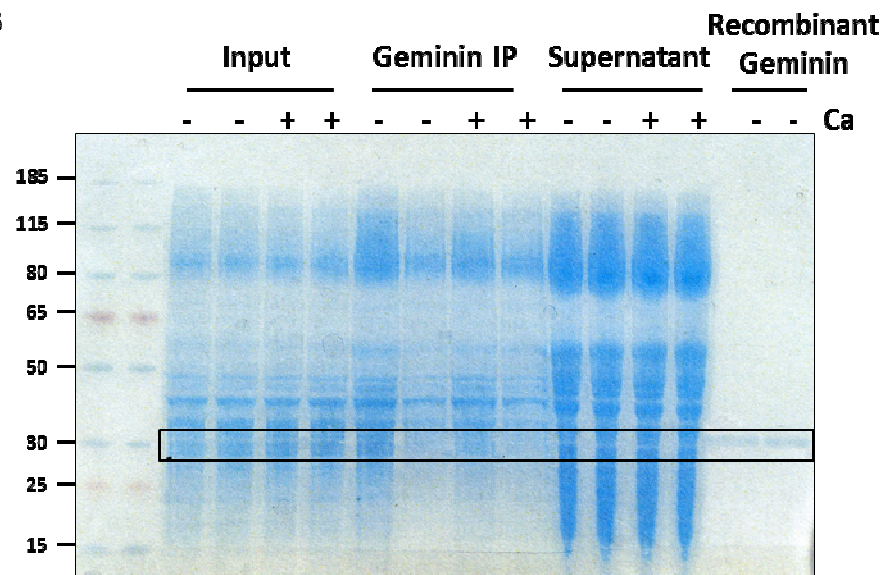
A**B**

Figure 33: Samples for Mass Spectrometry. (A) Control western shows the activity of samples used. Interphase geminin weakly interacts with Cdt1 showing that it is mostly inactive. (B) Coomassie stain of samples for mass spectrometry analysis. Boxed area shows slices that were processed by in gel digestion of proteins.

A Metaphase Input

Geminin H 12/219 Amino acids (5.47% Coverage)
MNTNKKQRLD MEKPTMSIKN YFVDK**TNESL** **APRR**TLKVIQ
PSASGCLVGR TKEPVKNSTK RKLWNDQLTS KKAKVEVAVD
PEHQENKDCS SEAYDLMVKE TPTCLYWKEV AEERRKALYE
ALQENEKLHK EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PRSLEDLKDL DLEEARFEDE ADMAEARIED
ETDMARPSNS DQNMDAHTV

Geminin L 13/216 Amino acids (6.01% Coverage)
MNSNMKQRS D VENPSMSIKN YIVDKTNEAL **APRR**TLKVIQ
QSASGCLVGR TKEPAKNSTK RKLWNDQLTS KKAKVEVAVD
PEHQENKDCP SEAYDLMVKE TPTCLYWKDV AEERRKALYE
ALQENEKLHQ EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIER**LTGNA** **PQSLED**LK**NL** DLEEARFEDE AESRIEDET
MTQPSSSDQN MDKQTV

B Metaphase Flow through

Geminin H 42/219 Amino acids (19.18% Coverage)
MNTNKKQRLD **MEK**PTMSIKN YFVDKTNE**SL** **APRR**TLK**VIQ**
PSASGCLVGR **TKEPVKNSTK** **RKLW**NDQLTS **KKAK**VEVAVD
PEHQENKDCS SEAYDLMVKE TPTCLYWKEV AEERRKALYE
ALQENEKLHK EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PRSLEDLKDL DLEEARFEDE ADMAEARIED
ETDMARPSNS DQNMDAHTV

Geminin L 33/216 Amino acids (15.28% Coverage)
MNSNMKQRS D VENPSMSIKN YIVDKTNEAL **APRR**TLKVIQ
QSASGCLVGR TKEPAKNSTK RKLWNDQLTS KKAKVEVAVD
PEHQENKDCP SEAYDLMVKE TPTCLYWKDV AEERR**KALYE**
ALQENEKLHQ **EIELKDEEIA** RLKQENDELM ELAGHVQYMA
NMIER**LTGNA** **PQSLED**LK**NL** DLEEARFEDE AESRIEDET
MTQPSSSDQN MDKQTV

Figure 34: Coverage of Geminin Peptides in Metaphase Inputs and Flowthrough. The coverage of peptides from metaphase (A) input and (B) flowthrough samples digested with trypsin (Blue) and Lys-C (Red) are highlighted. Motifs that regulate protein stability, mediate Cdt1 interaction and make up the coiled-coil are underlined. There was low coverage of geminin H and geminin L in both metaphase input and flowthrough.

A Interphase Input

Geminin H 46/219 Amino acids (21% Coverage)
MNTNKKQRLD MEKPTMSIKN YFVDKTNESL APRRTLKVIQ
PSASGCLVGR TKEPVKNSTK RKLWNDQLTS KKAKVEVAVD
PEHRENKDCS SEAYDLMVKE TPTCLYWKEV AEERRKALYE
ALQENEKLHK EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PRSLEDLKD L DLEEARFEDE ADMAEARIED
ETDMARPSNS DQNMDAHTV

Geminin L 54/216 Amino acids (25% Coverage)
MNSNMKQ~~RS~~D VENPSMSIKN YIVDKTNEAL APRRTLKVIQ
QSASGCLVGR TKEPAKNSTK RKLWNDQLTS KKAKVEVAVD
PEHQENKDCP SEAYDLMVKE TPTCLYWKDV AEERRKALYE
ALQENEKLHQ EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PQSLEDLKNL DLEEARFEDE AESRIEDET
MTQPSSSDQN MDKQTV

B Interphase Flow through

Geminin H 128/219 Amino acids (58.45% Coverage)
MNTNKKQRLD MEKPTMSIKN YFVDKTNESL APRRTLKVIQ
PSASGCLVGR TKEPVKNSTK RKLWNDQLTS KKAKVEVAVD
PEHRENKDCS SEAYDLMVKE TPTCLYWKEV AEERRKALYE
ALQENEKLHK EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PRSLEDLKD L DLEEARFEDE ADMAEARIED
ETDMARPSNS DQNMDAHTV

Geminin L 25/216 Amino acids (11.57% Coverage)
MNSNMKQ~~RS~~D VENPSMSIKN YIVDKTNEAL APRRTLKVIQ
QSASGCLVGR TKEPAKNSTK RKLWNDQLTS KKAKVEVAVD
PEHQENKDCP SEAYDLMVKE TPTCLYWKDV AEERRKALYE
ALQENEKLHQ EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PQSLEDLKNL DLEEARFEDE AESRIEDET
MTQPSSSDQN MDKQTV

Figure 35: Coverage of Geminin Peptides in Interphase Input and Flowthrough. The coverage of peptides from interphase (A) input and (B) flowthrough samples digested with trypsin (Blue) and Lys-C (Red) are highlighted. Overlap of peptides identified by both enzymes is shown in purple. Motifs that regulate protein stability, mediate Cdt1 interaction and make up the coiled-coil are underlined. There was low coverage of geminin H and geminin L in interphase input sample and geminin L in the flowthrough sample. However, there was moderate coverage of geminin H in the interphase flowthrough.

A Metaphase Geminin IP

Geminin H 188/219 Amino acids (85.85% Coverage)
MNTNKKQRLD MEKPTMSIKN YFVDKTNESL APRRTLKVIQ
PSASGCLVGR TKEPVKNSTK RKLWNDQLTS KKAKVEVAVD
PEHRENKDCS SEAYDLMVKE TPTCLYWKEV AEERRKALYE
ALQENEKLHK EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PRSLEDLKDL DLEEARFEDE ADMAEARIED
ETDMARPSNS DQNMDAHTV

Geminin L 192/216 Amino acids (88.88% Coverage)
MNSNMKQRS D VENPSMSIKN YIVDKTNEAL APRRTLKVIQ
QSASGCLVGR TKEPAKNSTK RKLWNDQLTS KKAKVEVAVD
PEHQENKDCP SEAYDLMVKE TPTCLYWKDV AEERRKALYE
ALQENEKLHQ EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PQSLEDLKNL DLEEARFEDE AESRIEDET
MTQPSSSDQN MDKQTV

B Interphase Geminin IP

Geminin H 163/219 Amino acids (74.43% Coverage)
MNTNKKQRLD MEKPTMSIKN YFVDKTNESL APRRTLKVIQ
PSASGCLVGR TKEPVKNSTK RKLWNDQLTS KKAKVEVAVD
PEHRENKDCS SEAYDLMVKE TPTCLYWKEV AEERRKALYE
ALQENEKLHK EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PRSLEDLKDL DLEEARFEDE ADMAEARIED
ETDMARPSNS DQNMDAHTV

Geminin L 167/216 Amino acids (77.31% Coverage)
MNSNMKQRS D VENPSMSIKN YIVDKTNEAL APRRTLKVIQ
QSASGCLVGR TKEPAKNSTK RKLWNDQLTS KKAKVEVAVD
PEHQENKDCP SEAYDLMVKE TPTCLYWKDV AEERRKALYE
ALQENEKLHQ EIELKDEEIA RLKQENDELM ELAGHVQYMA
NMIERLTGNA PQSLEDLKNL DLEEARFEDE AESRIEDET
MTQPSSSDQN MDKQTV

Figure 36: Coverage of Geminin Peptides in Metaphase and Interphase IPs. The coverage of peptides from (A) metaphase and (B) interphase geminin IP samples digested with trypsin (Blue) and Lys-C (Red) are highlighted. Overlap of peptides identified by both enzymes is shown in purple. Motifs that regulate protein stability, mediate Cdt1 interaction and make up the coiled-coil are underlined. There significant coverage of geminin H and geminin L in metaphase IPs and good coverage of proteins in interphase IPs.

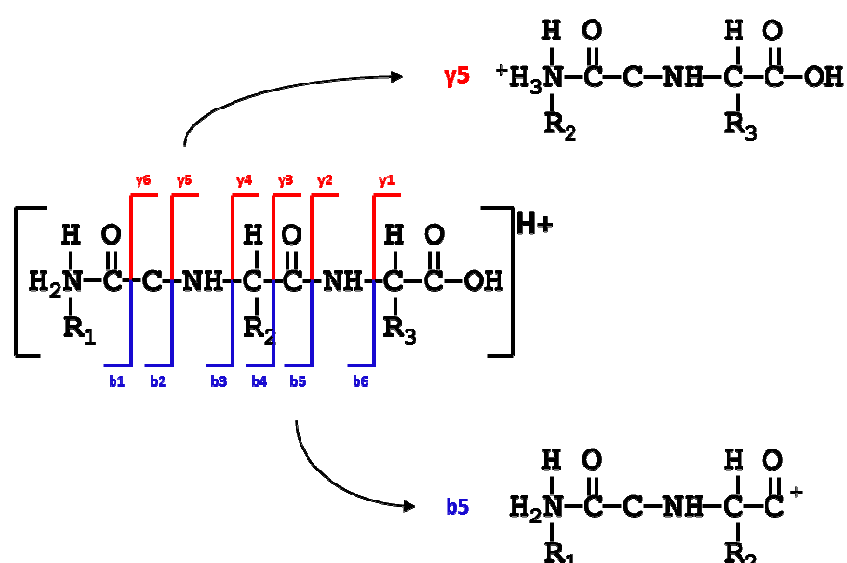
Peptide and PTM identification was performed by two programs which use different search algorithms, MaxQuant and PEAKS. MaxQuant matches the m/z of peptides to a reference database and searches for spectra of identified peptides with user specified modifications. PEAKS uses de novo sequencing to search all peptides for all modifications and although it uses a reference database initially, that is not absolutely required for peptide identification. A number of acetylated and methylated peptides were identified using MaxQuant (Table 4), however none were identified with PEAKS. In addition a greater coverage of peptides was observed in MaxQuant. As there was no concordance between MaxQuant and PEAKS in the identification of modifications, the spectra for each modified peptide is shown to further support their existence (Figure 38). The spectra plot the relative abundance and m/z of b and y ions. B and y ions show where the bonds have fragmented along the amino acid backbone (Figure 37). Fragments that retain the charge on the N-terminal fragment are b ions and y ions retain the charge on the C-terminus.

Geminin is an APC/C target due to the presence of a D-box motif. An additional ubiquitin initiation motif consisting of the residues ⁵⁰RTK-KRK⁶² makes geminin a more efficient substrate of the APC/C (Benjamin et al., 2004, Williamson et al., 2011). R50-methylation, K52-methylation and K62-acetylation were identified on peptides from metaphase geminin IPs. This suggests that geminin stability may be regulated by the post-translational modification of the ubiquitin initiation motif. R50-methylation and K52-methylation were, however, also detected in interphase IPs, although they were much less abundant as only 2% of peptides were modified. A mutational analysis of geminin it has previously shown that the mutation of K60 and K62 to alanine partially stabilised recombinant geminin in extracts, whereas a combination of R50, K52, K60

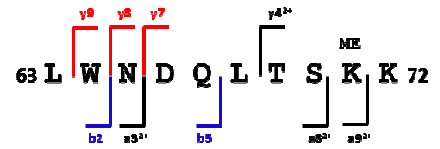
and K62 mutants resulted in a complete stabilisation of recombinant geminin (Benjamin et al., 2004). K116 is a highly conserved residue located in the coiled-coil region of

Geminin		Modified sequence	Acetyl Methyl (K) (K/R)		Enzyme	Stage	Sample	Mod Ratio
1	L	$_7\text{QR(m)SDVENPSMSIKNYIVDK(m)}_{25}$	0	2	Tryp	Inter	Input	1
2	L	$_{116}\text{K(m)ALYEALQENЕК}_{127}$	0	1	Tryp	Inter	Input	0.008
3	L	$_{116}\text{K(m)ALYEALQENЕК}_{127}$	0	1	Tryp	Inter	Input	1.99
4	L	$_{63}\text{LWNDQLTSK(m)K}_{72}$	0	1	Tryp	Inter	Input	1
5	L	$_{63}\text{K(ac)LWNDQLTSK}_{71}$	1	0	Tryp	Meta	IP	0.11
6	H	$_{38}\text{VIQPSASGCLVGR(m)}_{50}$	0	1	Tryp	Meta	IP	0.04
7	L	$_{38}\text{VIQQSASGCLVGR(m)TK(m)}_{52}$	0	2	Lys	Meta	IP	0.62
8	L	$_{116}\text{K(m)ALYEALQENЕК}_{127}$	0	1	Tryp	Meta	IP	2.56
9	L	$_{62}\text{KLWNDQLTSK(m)K(m)}_{72}$	0	1	Tryp	Inter	IP	1
10	L	$_{38}\text{VIQQSASGCLVGR(m)TK(m)}_{52}$	0	2	Lys	Inter	IP	0.02
11	L	$_7\text{QRSDVENPSMSIK(m)}_{19}$	0	1	Tryp	Inter	IP	1
12	H	$_2\text{NTNK(ac)K(m)QR(m)LDMEK}_{13}$	1	2	Lys	Meta	FT	1
13	H	$_2\text{NTNK(ac)K(m)QR(m)LDMEK}_{13}$	1	2	Lys	Inter	FT	1
14	L	$_{116}\text{K(m)ALYEALQENЕК}_{127}$	0	1	Tryp	Inter	FT	1

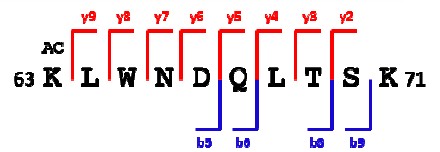
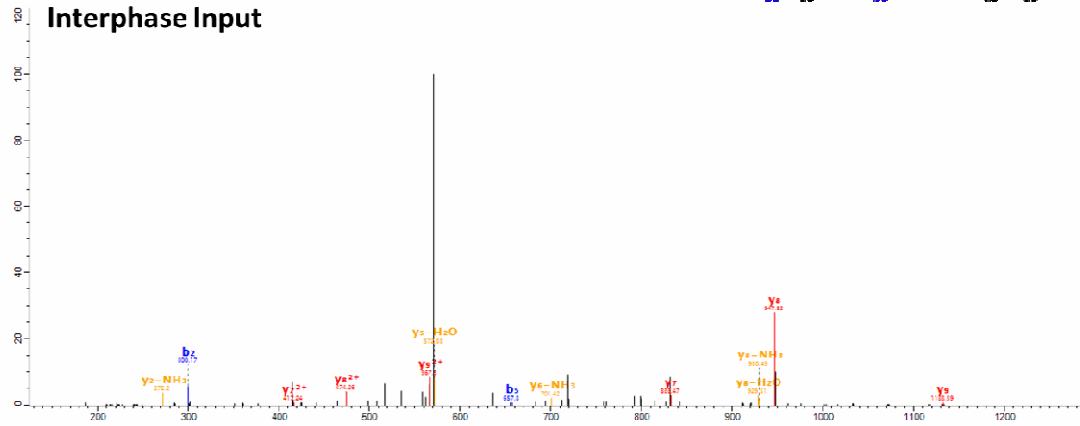
Table 4: Modified Geminin Peptides Identified by Mass Spectrometry. The Mod Ratio refers to the ratio of modified peptide intensity to total intensity for that peptide. Modified peptides identified in immunoprecipitation samples are highlighted in bold. Peptide 9 methylation is shown in italics: the peptide is monomethylated either on K71 or K72



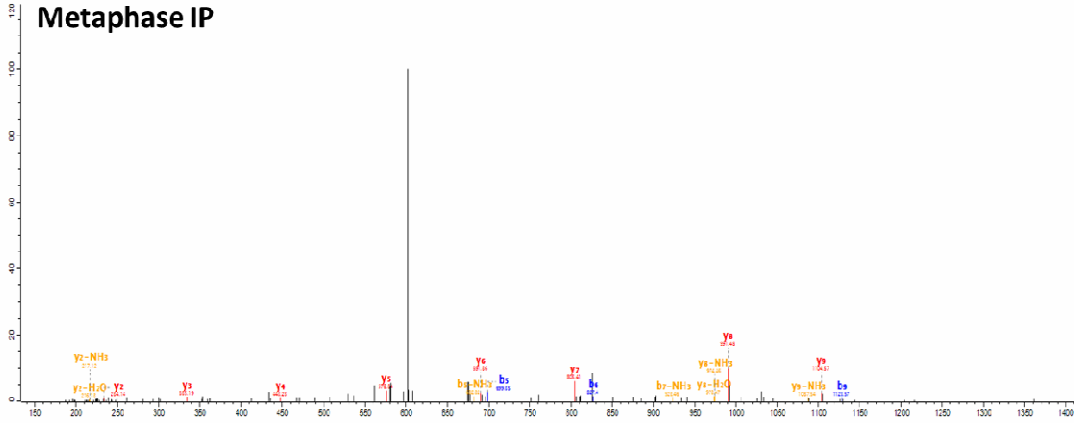
[illegible]

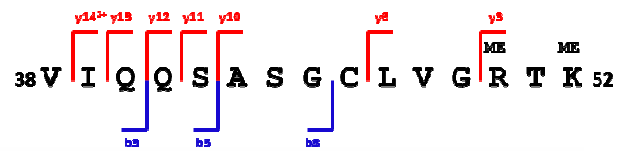


Peptide 4
Interphase Input

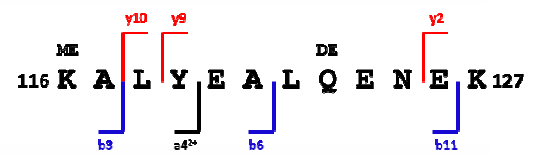
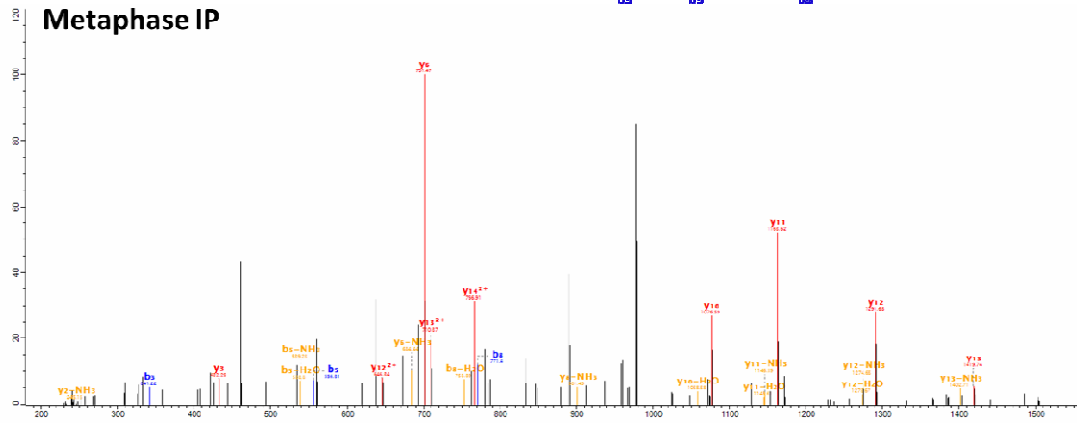


Peptide 5
Metaphase IP

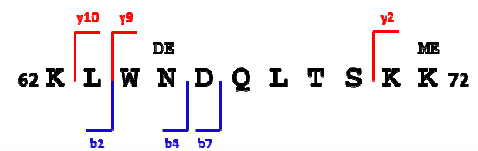
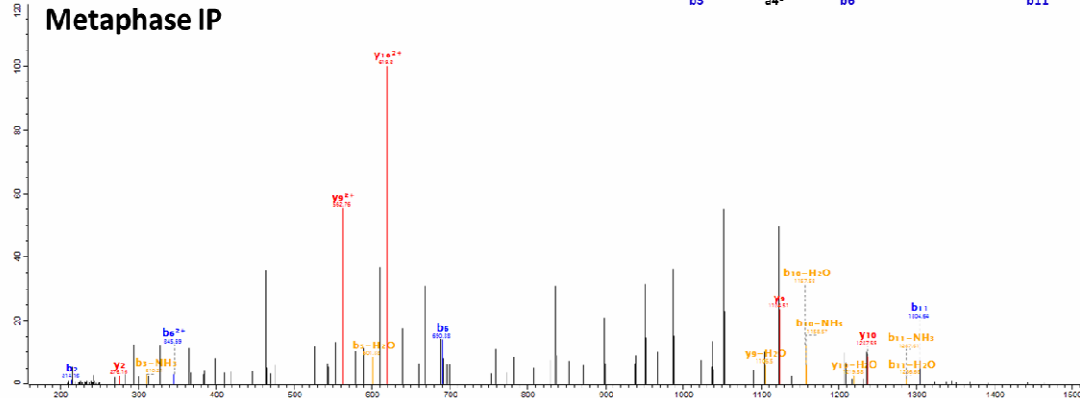




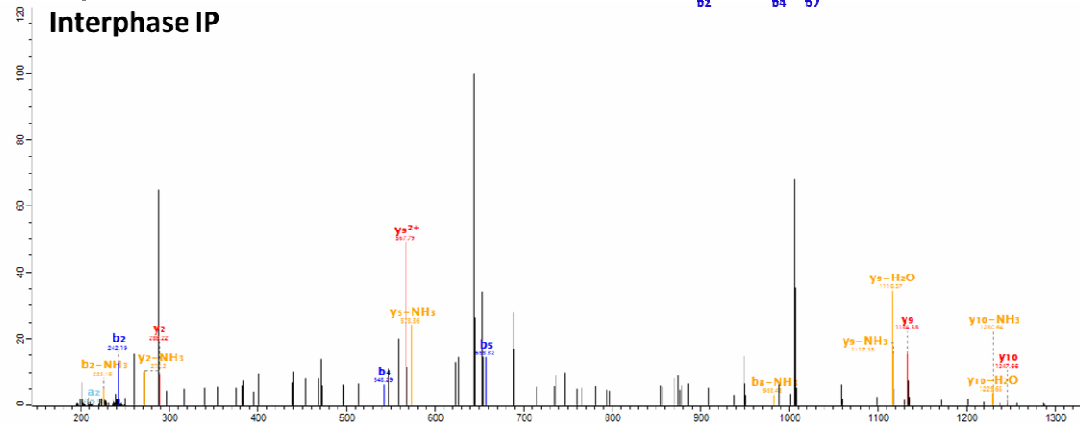
Peptide 7
Metaphase IP

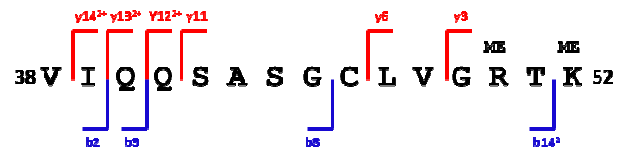


Peptide 8
Metaphase IP

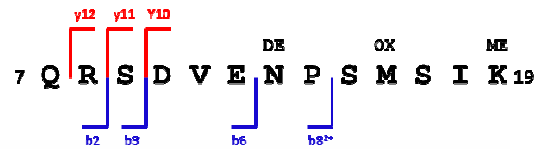
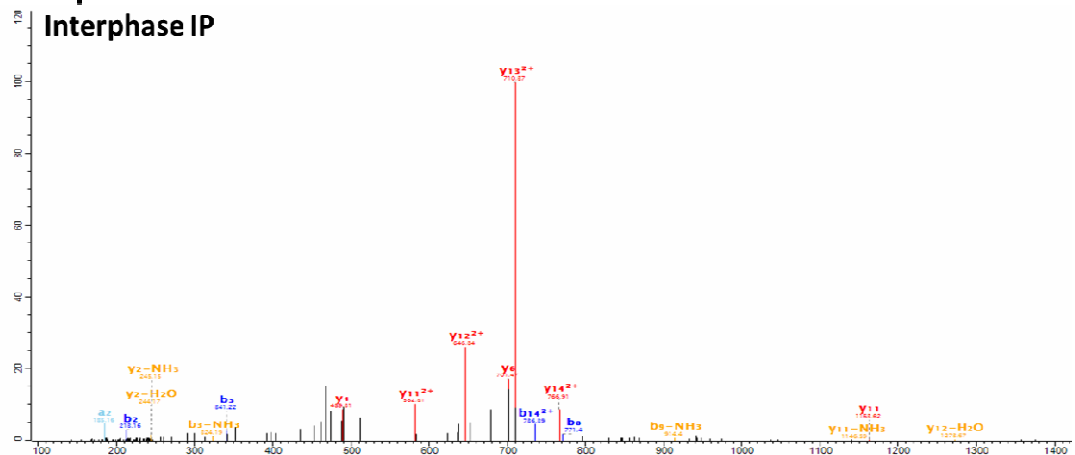


Peptide 9
Interphase IP

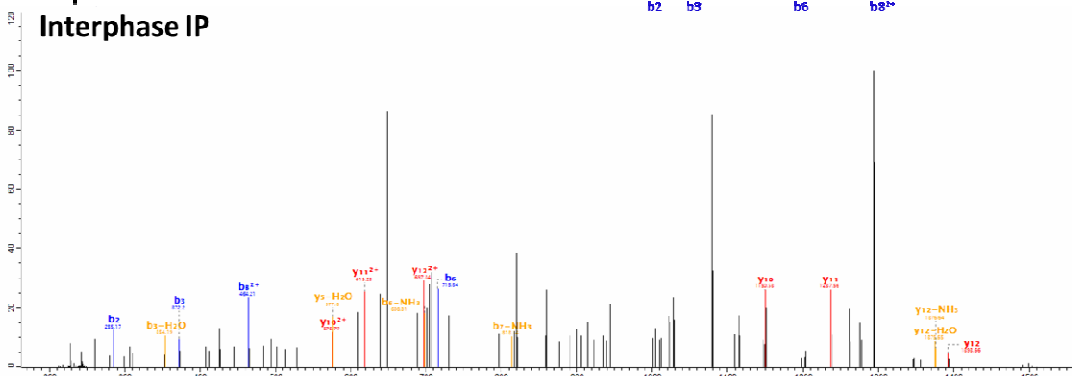




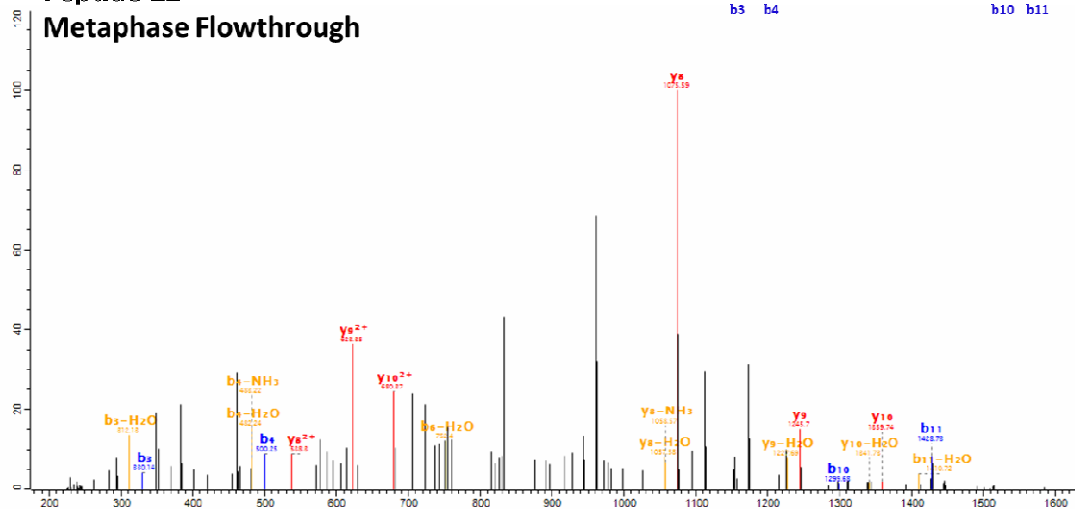
Peptide 10
Interphase IP

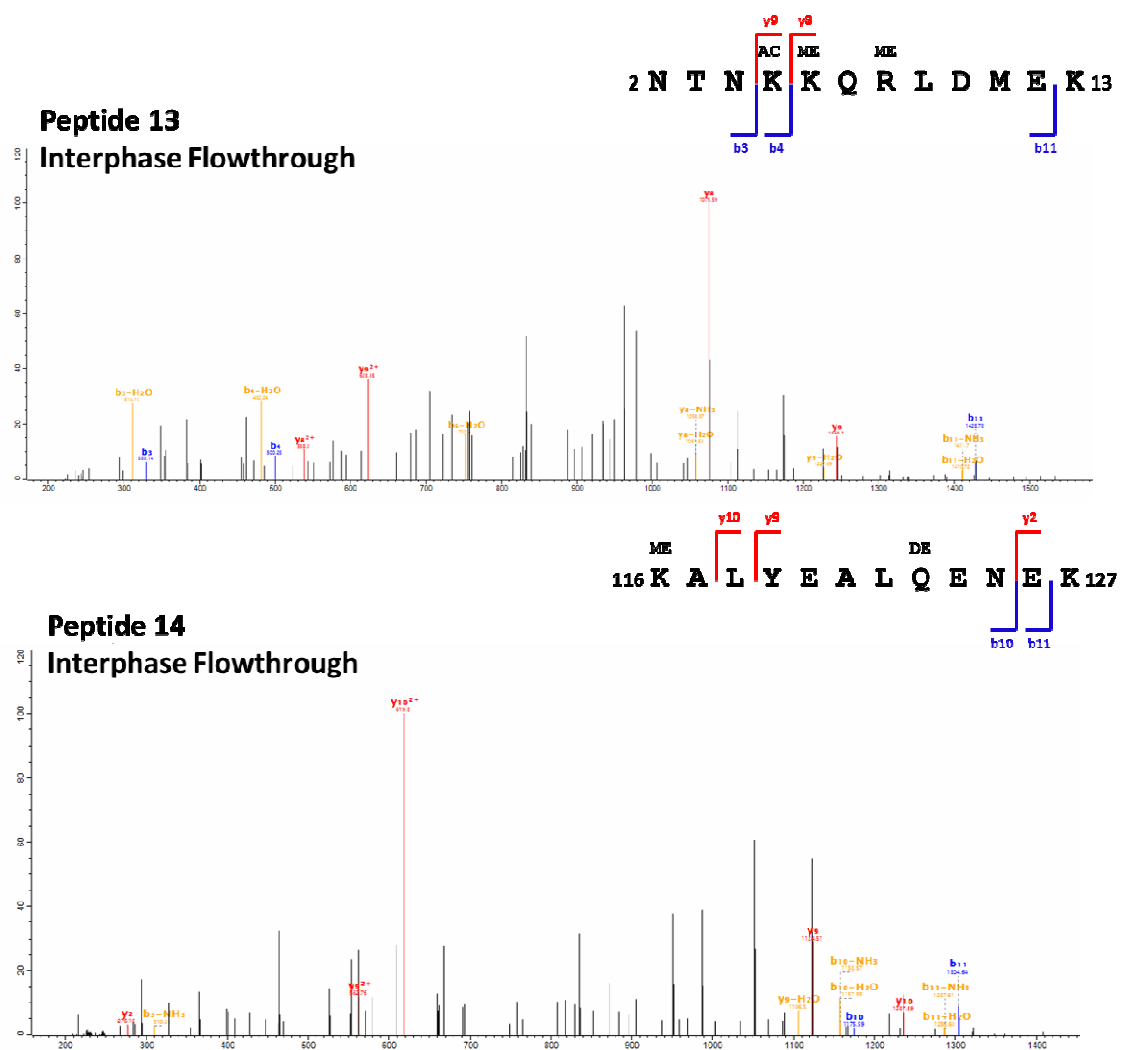


Peptide 11
Interphase IP



Peptide 12
Metaphase Flowthrough





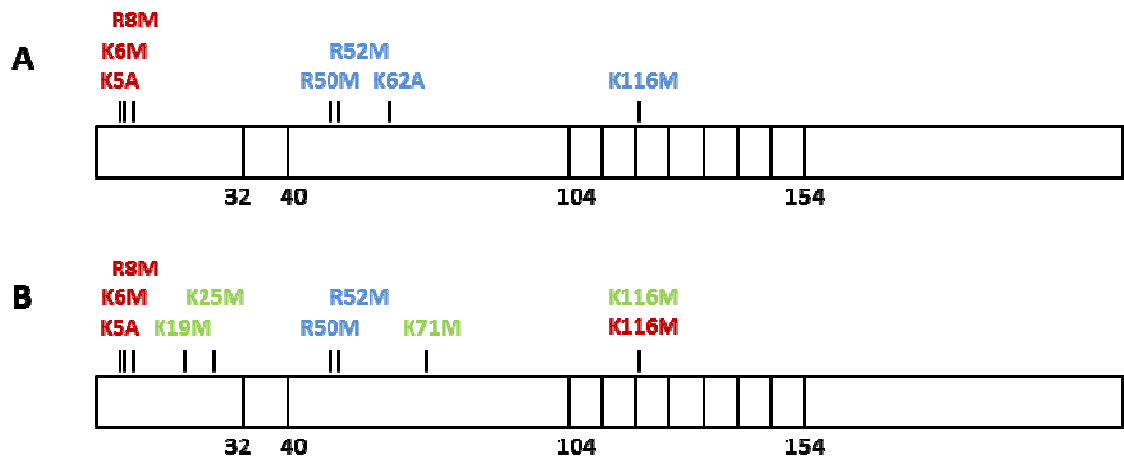


Figure 39: Sites of Geminin Post-translational Modifications. The positions of residues modified by post translational modification on peptides from (A) metaphase and (B) interphase extract. 32-40 shows the position of the D-box. 104-154 shows the position of the coiled-coil. A=acetylation M=Methylation. The modifications were highlighted in blue for IPs, red for flowthroughs and green in inputs.

geminin within the primary Cdt1 interaction site (Figure 6) (De Marco et al., 2009). This could hold potential as a key site for regulating the ability of geminin to interact with Cdt1, however the K116 methylation was abundant in both metaphase and interphase samples. The methylation of K5, K6 and R8 was observed in the flowthrough in both metaphase and interphase, suggesting that proteins containing these modifications may be less bind less efficiently to the geminin antibody during the IP.

The function of these modifications cannot be speculated upon as the extreme N-terminus of geminin has no known functions, however the residues here are very well conserved. K71, which is part of a proposed nuclear localisation signal domain (Boos et al., 2006), was methylated in interphase. This is not likely to be playing a role in the regulation of geminin function. Residues K19 and K25, which are situated close to the D-box motif, were also methylated suggesting they may potentially play a role in stabilisation of the protein.

No modifications were identified in key domains that would be predicted to have major implications on the ability of geminin to interact with Cdt1. However, although this

experiment made improvements in identifying geminin peptides by mass spectrometry, it has not been exhaustive in showing that geminin activity is not regulated by post-translational modification. To come to a conclusion on this mechanism of inactivation a complete coverage of the geminin proteins is required. In these experiments a key residue located between the primary and secondary Cdt1 interaction sites (residues 108-115 of geminin H) is missing from all samples. This is consistent with all other mass spectrometry carried out in the lab. A modification here could potentially play a role in modulating geminin activity. This peptide was identified in the recombinant geminin control sample, suggesting that this peptide may not be detected due to some form of modification. A large peptide covering residues 144-165 of geminin H is identified in metaphase, but not in interphase samples. The region contains geminin's tertiary Cdt1 interaction site. This site has been shown to mediate the formation of a geminin-Cdt1 heterohexamer (De Marco et al., 2009) and it has been hypothesised that hexamer formation is required for the inhibition of Cdt1. Therefore, a modification of this region by PTM could potentially regulate geminin activity. Identification of these peptides specifically in metaphase and not in interphase suggests that may not be identified due to a modification.

4.7 A Potential Role for SUMO

The preparation of peptides for mass spectrometry requires protein digestion with trypsin or another protease. The digestion of a SUMOylated protein removes the SUMO from the target protein and this leaves no detectable mark. Therefore if geminin was targeted for SUMOylation, it would not be detectable by mass spectrometry. It has been reported that Geminin can interact with the SUMO-specific proteases/isopeptidases SENP1 and SENP2 in human cells (Gardner et al., 2011). This suggests that geminin may be a target for sumoylation. Geminin sequences were analysed with SUMO site prediction software (<http://sumosp.biocuckoo.org/online.php>) and two potential

consensus sites were identified in *Xenopus* geminin H and L and a single site in human geminin (Figure 40A). These sites are both located within the coiled-coil region, suggesting that should they exist they could potentially play a role in regulating geminin activity.

In vitro sumoylation reactions were carried out with either SUMO1, SUMO2, or SUMO2-T90K and wild type recombinant geminin. SUMO2-T90K was included as this modification allows for the detection of the modified site by mass spectrometry. Samples were analysed by SDS-PAGE followed by Coomassie staining. Formation of higher molecular weight conjugates shows that geminin can be a substrate for mono-sumoylation and the addition of sumo chains *in vitro*. In a two hour reaction the levels of sumoylated geminin were relatively low compared to the control reaction carried out with the known target, IRF2 (Figure 40B and C). The majority of modified geminin in this reaction was present in a mono-sumoylated form. After a 4 hour incubation SUMO chains were added onto recombinant geminin (Figure 41). These bands were shown to be geminin specific by western blot analysis. This confirms that geminin is a potential SUMO substrate.

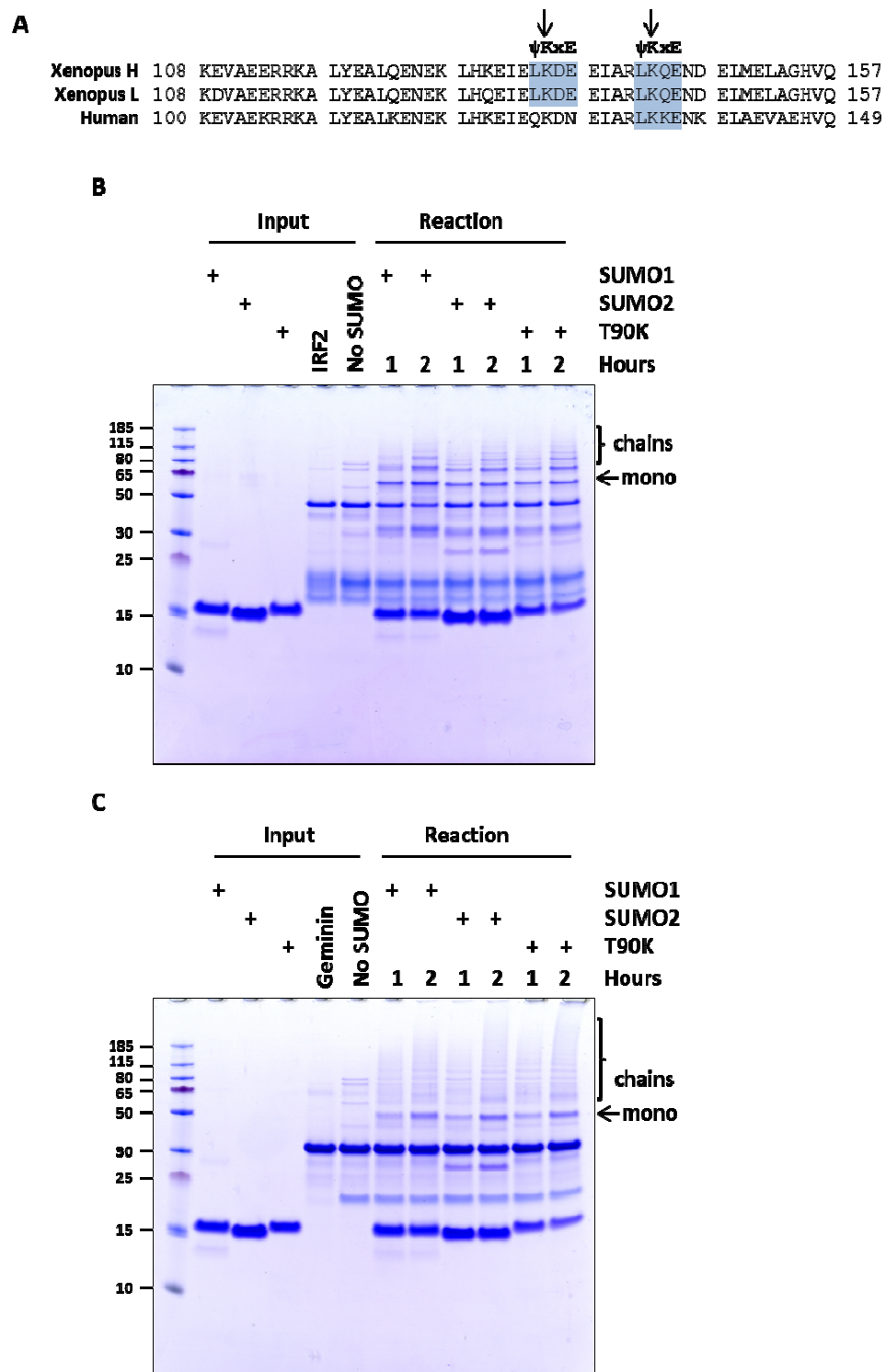


Figure 40: Geminin is SUMOylated *in vitro*. (A) Identification of two potential SUMOylation consensus sites (ψKxE) in *Xenopus* geminin H and L, once of which is conserved in human cells. Both sites are located within the coiled-coil of geminin. SUMOylation assays carried out for 2 hours with SUMO1, SUMO2 and SUMO2-T90K in a control reaction of a known SUMO target IRF2 (B) or geminin (C). After 2 hours samples were analysed by SDS-PAGE and coomassie staining.

4.8 Summary- APC/C mediates a Licensing switch and geminin is modified by PTMs

The aim of this chapter was to gain insights into the molecular events that occur downstream of APC/C activation that result in the inactivation of geminin and to determine whether geminin is being regulated by post translation modification. A threshold of APC/C activity is required for the inactivation of geminin, which take 6-8 or 8-10 minutes, after which time the APC/C is no longer required. This APC/C activity is likely to mediate regulation of geminin by methods other than ubiquitination as there is a pool of geminin that is not modified by ubiquitin during the exit from metaphase arrest and this portion of geminin can be reactivated in the nucleus in the absence of DUB activity. Multiple post-translational modifications of geminin H and L were identified by mass spectrometry after IP protocols were optimised to provide samples relatively quickly. The identified modifications potentially result in the stabilisation of geminin. PTMs in regions which would be expected to alter the function of geminin were not identified; however key regions of interphase geminin were not covered in the experiment. In addition, geminin has been shown to be a potential target of SUMOylation.

5.0 Regulation of Geminin by Protein-protein Interactions

5.1 Introduction

The binding of an inhibitor could sequester geminin, freeing Cdt1, to allow for licensing activity after the metaphase-anaphase transition. A number of geminin interacting proteins have been reported and the best characterised of these are the interactions of geminin with coiled-coil proteins that function during cell fate decisions. Pre-incubation of recombinant geminin with recombinant HoxA11 was found to reduce geminin-dependent Cdt1 pulldown from cell extracts, suggesting HoxA11 competes with Cdt1 for geminin binding (Luo et al., 2004). Overexpression of HoxB7 or HoxA11 in mouse embryonic fibroblasts also reduced the amount of Cdt1 that co-immunoprecipitates with geminin (Luo et al., 2004). In addition, high levels of *Xenopus* Six3 or Six6 was shown to compete with recombinant Cdt1 for binding to GST-geminin *in vitro* (Del Bene et al., 2004). These reports lend support to a mechanism where activation of a geminin inhibitor protein at the metaphase-anaphase transition could preferentially bind to geminin and result in the activation of licensing. However, these specific interactions are unlikely to account for geminin inactivation in *Xenopus* egg extracts as these transcription factors are not expressed until later in development. Thus it is of interest to determine whether geminin is bound by novel proteins during the activation of licensing in *Xenopus* egg extracts.

An alternative protein-protein interaction mediated mechanism has been described where geminin-Cdt1 complexes self-associate into larger complexes with specific stoichiometries that determine the activity of the complex. This model predicts that geminin-Cdt1 complexes can exist in multiple forms. There is evidence to suggest that a heterohexameric complex (geminin:Cdt1 at 4:2 ratio) is inhibited for licensing activity

and a heterotrimer (geminin:Cdt1 at 2:1 ratio) is permissive for licensing (De Marco et al., 2009). An equilibrium between these complexes could coordinate a stoichiometric switch mechanism to regulate licensing. This switch could be mediated by changing the absolute concentrations of geminin and Cdt1 or by modifying key interaction sites. There is biochemical evidence to support the existence of a permissive complex; a co-expressed and purified geminin^{DEL}-Cdt1 complex was shown to retain licensing activity similar to Cdt1 alone when added to interphase extracts (Lutzmann et al., 2006). Therefore, this model requires validation with endogenous proteins alone and to determine the stoichiometry of geminin and Cdt1 complexes at different stages of the cell cycle in egg extracts.

The main aim of this chapter is to investigate the potential role of protein-protein mediated interactions in the regulation of geminin activity. Experiments were undertaken to provide evidence for or against either of the hypothesised mechanisms: the sequestration of geminin by an inhibitor or the stoichiometric switch of geminin-Cdt1 complexes.

5.2 Endogenous Geminin-Cdt1 Complex Size and Stability

Fractionation of metaphase and interphase extracts by size exclusion chromatography has shown that geminin elutes into four potentially distinct peaks (Figure 13). Whereas both of the metaphase complexes are capable of interacting with Cdt1, neither of the 2 complexes identified in interphase extracts are competent to bind to Cdt1 (Figure 16). It has been shown that geminin becomes reactivated upon nuclear import (Hodgson et al., 2002) and this has been hypothesised to increase the local concentration of geminin to drive the formation of geminin-Cdt1 complexes that are not permissive for licensing (De Marco et al., 2009). Geminin has been predicted to cooperatively bind to Cdt1 to form multimers in the nucleus lending support to the stoichiometric switch hypothesis

(Ode et al., 2011). This model predicts the formation of a large complex in the nucleus. Experiments were undertaken to observe the reformation of endogenous geminin and Cdt1 complexes in the nucleus (Figure 42). It was important to determine if unique or nucleus-specific geminin-Cdt1 inhibitory complexes were formed or whether all inhibitory geminin-Cdt1 complexes in egg extracts form the same complex that is present in metaphase. The observation of larger complexes would support the hypothesis that multimers of geminin-Cdt1 form in the nucleus to provide inhibitory activity.

Pre-incubated extract (PIE) was prepared by allowing nuclei to assemble in extract for 45 minutes, after which time extract was diluted and centrifuged, spin-crushing the nuclei and leaving geminin in the supernatant (Hodgson et al, 2002). During the incubation, nuclear assembly and nuclear protein import occurs, resulting in the reactivation of geminin. PIE was fractionated by size exclusion chromatography and analysed by western blot for geminin and Cdt1 (Figure 41A- 45 minutes). Samples immediately after the void volume were included to ensure no high molecular weight complexes were excluded from the analysis. Whereas the majority of geminin in this 45 minute sample elutes at a low molecular weight indicative of inactive protein, all the Cdt1 identified co-fractionates with geminin between 669 and 443 kDa, similar to a metaphase geminin-Cdt1 complex, suggesting that a portion of geminin was reactivated sufficiently quickly to inhibit Cdt1 at the start of S-phase. This data suggests that metaphase and nuclear inhibitory geminin-Cdt1 complexes are likely to consist of the same ratio of geminin and Cdt1.

To further investigate the size of nucleoplasmic geminin and Cdt1 complexes, a 'Nucleoplasmic Extract' (NPE)(Walter et al., 1998) was prepared. In this procedure, nuclei assembled for 90 minutes are carefully floated in extract, so that intact nuclei are

separated from the cytoplasm before spin-crushing is performed. NPE was fractionated by size exclusion chromatography to observe nuclear geminin-Cdt1 complex formation (Figure 42B). Evidence of a metaphase-like geminin-Cdt1 complex in NPE would further support that idea that only a single inhibitory complex is formed in extracts. However, NPE was found to contain little or no Cdt1, as it is presumably subjected to replication-coupled destruction during the 90 minute reaction. Therefore, NPE could not be used to identify inhibitory geminin-Cdt1 complexes. Interestingly, however, geminin eluted into a single peak between 443 and 200 kDa, similar to the lower molecular weight peak of metaphase geminin, suggesting that this complex consists of reactivated geminin.

In addition to investigating the size of nuclear geminin-Cdt1 complexes, PIE was generated at two timepoints, 45 and 90 minutes, and analysed by size exclusion chromatography to give a rough estimation of the kinetics of geminin reactivation (Figure 42A). The inhibitory geminin-Cdt1 complexes migrating between 669 and 443 kDa, is lost over time, as is the case in NPE, presumably due to the DNA replication dependent destruction of Cdt1. At 45 minutes the bulk of geminin remains in a ~150 kDa complex, comparable to interphase inactive geminin. By 90 minutes this lower peak of geminin shifted to a higher molecular weight at ~250 kDa, similar to the peak of uncomplexed active geminin observed in metaphase extract. This suggests that the bulk of geminin became reactivated from 45-90 minutes during the later stages of DNA replication. It is hypothesised that geminin nuclear import follows quickly after nuclear formation to ensure Cdt1 is inhibited before CDKs reach a threshold of activity capable of initiating DNA replication (Hodgson et al., 2002, Gillespie et al., 2007). In this context the slow kinetics of reactivation observed here (Figure 42A) are surprising as this suggests that the bulk of geminin is not immediately reactivated upon nuclear import, but requires a slower mechanism to reform an inhibitory complex. One caveat to

this observation, however, is that the kinetics of geminin nuclear import are not known, and therefore the 45 minute PIE may contain a mixture of active nuclear and inactive cytoplasmic geminin. The observed transition from a lower inactive complex to a higher active geminin complex may therefore reflect a continued import of geminin from the cytoplasm to the nucleus from 45-90 minutes. To investigate this point, NPE could be prepared at different times after nuclear addition, to distinguish between nuclear and cytoplasmic pools of geminin.

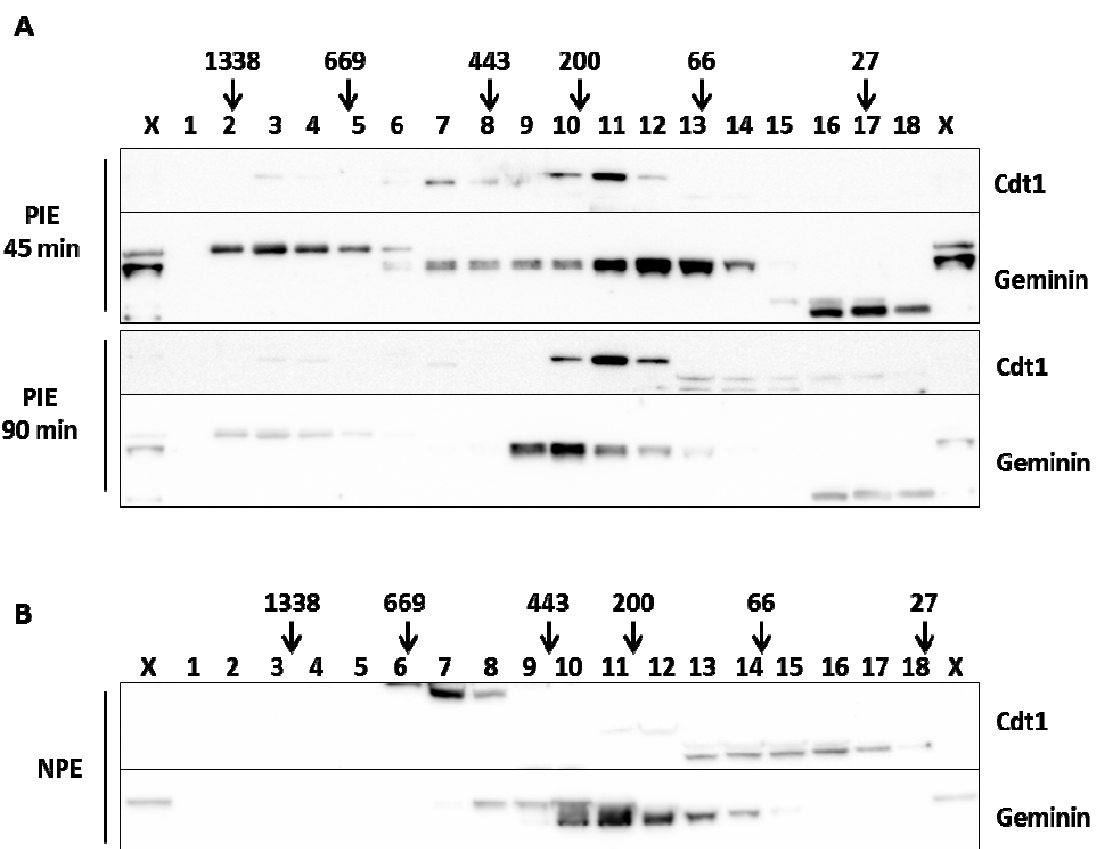


Figure 42: Fractionation of Geminin-Cdt1 Complexes in PIE and NPE.(A)Extracts were released into interphase for 15 minutes by calcium addition and supplemented with 10 ng/μl of DNA. Extracts were then incubated for 45 or 90 minutes. Nuclei were lysed by a 1/4 dilution with LFB2/50 supplemented with 0.1% Triton and centrifuged to remove the DNA. Supernatants were filtered through a 0.2μm membrane before being fractionated by size exclusion chromatography. Fractions were analysed by western blot for geminin and Cdt1. Specific Cdt1 bands are present in lanes 6, 7 and 8. A frequently observed non-specific band runs slightly larger than Cdt1 on SDS-PAGE in lanes 10, 11 and 12. **(B)** NPE (see Methods) was fractionated by size exclusion chromatography and fractions were analysed by western blot for geminin and Cdt1.

Inhibitory geminin-Cdt1 complexes elute on gel filtration at a high molecular weight between 669 and 443 kDa. Cdt1 is a 69.5 kDa protein and it elutes at approximately 443 kDa in interphase. Geminin H and L proteins are 25.3 kDa and 24.8 kDa respectively and presumably exist as homo- and/or hetero-dimers of approximately 50 kDa in egg extracts. However, geminin elutes at multiple sizes between 66-200 kDa, at 200 kDa and at 443 kDa depending on the cell cycle stage. As Cdt1 contains alpha helical regions and geminin forms an elongated coiled-coil, when subjected to gel filtration these proteins are expected to fractionate at molecular weights higher than those predicted by the molecular mass of the proteins alone. However, these high molecular weight elution profiles could also be indicative of complex formation with novel regulatory proteins.

To investigate the hypothesis that the sequestration of geminin by an inhibitor protein results in the activation of licensing, metaphase and interphase extracts were fractionated by size exclusion chromatography in the presence of increasing concentrations of salt (Figure 43). A salt titration could potentially interrupt charge-charge interactions, and a shift of a geminin complex to a larger elution volume might indicate the loss of a binding partner. Under normal running conditions of 200 mM NaCl, metaphase geminin and Cdt1 complexes fractionated as expected, however, the extract used contained a relatively low amount of lower molecular weight metaphase geminin compared to typical extracts. This however did not affect the elution profile of geminin in interphase extract and two geminin peaks are observed below 443 and 200 kDa, respectively (lanes 9 and 12). At higher salt concentrations the transfer of proteins onto the membrane during the western blot was impeded, however the peak elutions could still be observed at high exposures. Metaphase geminin-Cdt1 complexes were

unaffected by the addition of 500 mM NaCl. The second geminin peak however, shifted to the right at this salt concentration, with an extra peak appearing just below 200 kDa (shift from lane 9 to 12). Interphase Cdt1 also began to shift one fraction to the right at 500 mM NaCl (shift from lane 8 to 9). Interphase geminin elution was significantly affected at 500 mM NaCl with the only observed fraction eluting close to 66 kDa (shift from 9 and 12 to 13). The majority of complexes were not affected further by increasing salt to 1000 mM, however, interphase geminin complexes shifted further and eluted at and below 66 kDa (lane 14). Geminin and Cdt1 interact tightly by hydrophobic interactions and the inactive metaphase geminin-Cdt1 complexes at ~500 kDa were not affected by salt at any concentration. This is consistent with a ~500 kDa complex that consists of geminin and Cdt1 only, as charge-charge mediated interactions should be disrupted at high salt. This experiment also shows that all geminin complexes, except for metaphase geminin-Cdt1, are sensitive to salt to some extent.

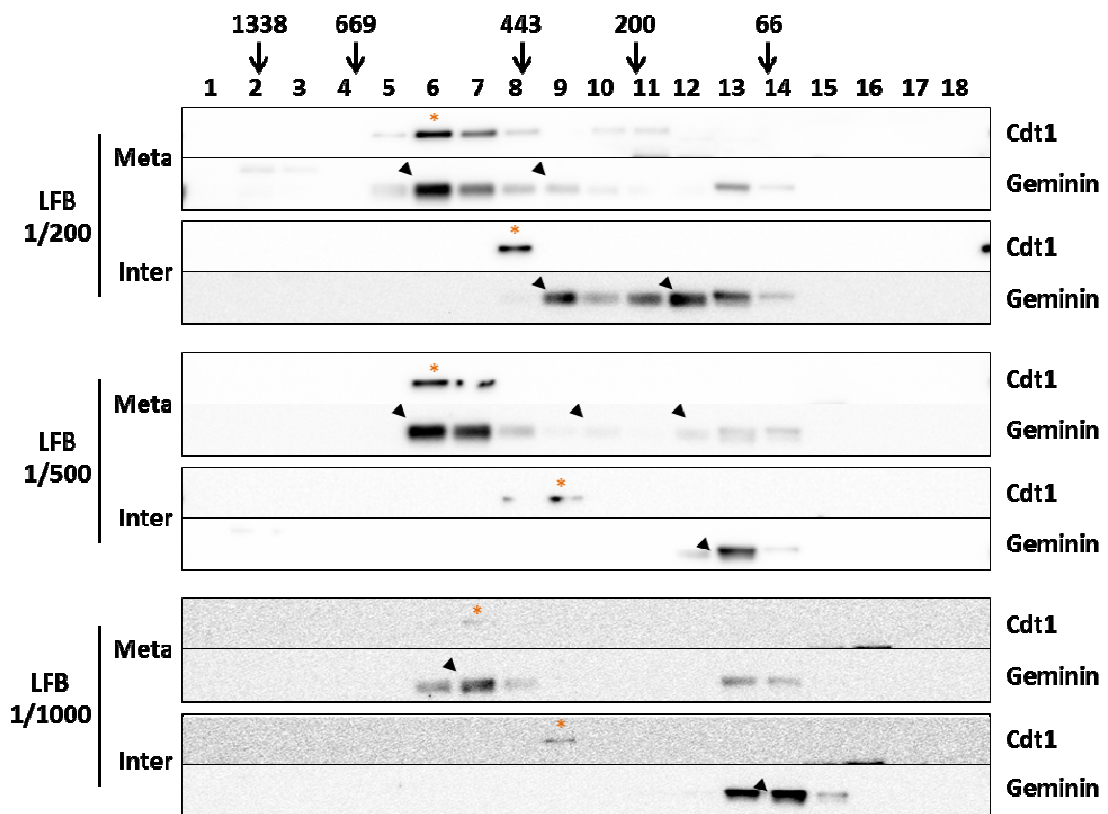


Figure 43: Geminin and Cdt1 Fractionation at High Salt. Metaphase and interphase extracts were diluted 1/5, filtered through a 0.2 μ m membrane and fractionated by size exclusion chromatography. Fractions were analysed by western blot for geminin and Cdt1. Orange asterisk indicates Cdt1 peak elution. Arrow indicates interphase geminin peak elution.

To gain more information about geminin complex stability and determine if the metaphase and interphase geminin complexes can interconvert, the peak geminin fractions were pooled and reapplied to the gel filtration column under the same conditions as the first round of fractionation (Figure 44). The more stable the geminin complexes are, the more likely they are to elute into the same fractions after the second run. Both metaphase peaks eluted at essentially the same size, demonstrating that these consist of stable complexes. Interphase complexes, however, were lost after being reapplied to the column, suggesting that they were unstable and took on a different form during the second fractionation. Potentially this could be a false negative if the protein concentration was simply too low for detection, however non-specific bands which were less abundant than geminin in the input (see lane 8 upper band) did elute into the

original fractions (interphase lane 9 and 10), suggesting the geminin peak was lost due to complex instability. This data, together with salt titration experiments, suggests that geminin not bound to Cdt1 may undergo dynamic charge-charge mediated interactions, either with itself or potentially with an unknown interacting protein.

5.3 Estimating the Molecular Weight of Geminin and Cdt1 Complexes

It appears that the metaphase inhibitory geminin-Cdt1 complex is likely to represent the largest stable form of geminin-Cdt1 multimers that form in egg extracts including both cytoplasmic and nuclear inhibitory complexes. In addition, fractionation of extracts in high salt did not alter the size of this complex suggesting it likely to consist of geminin and Cdt1 only. Optimisation of linear glycerol gradient sedimentation to resolve geminin and Cdt1 complexes was performed to allow for an estimation of the molecular weight of geminin and Cdt1 containing complexes using the Siegel and Monty equation (Erickson, 2009). This would in turn give information on the stoichiometry of endogenous geminin and Cdt1 complexes.

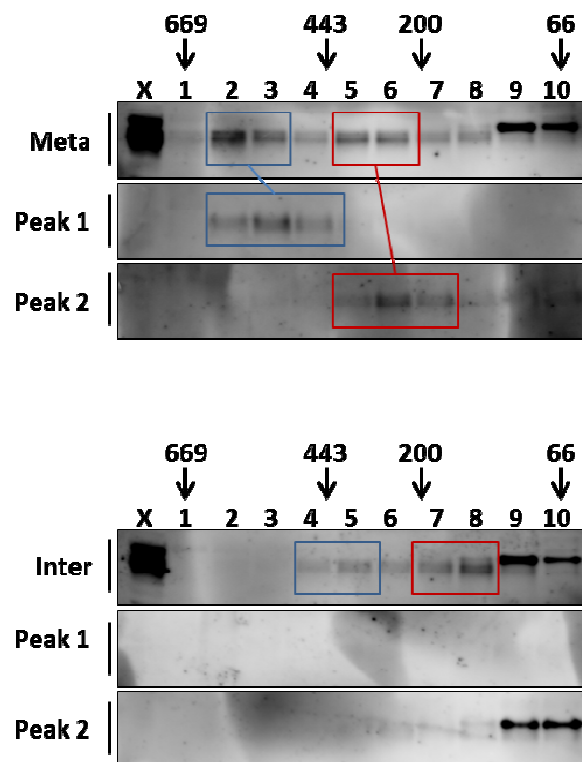


Figure 44: The Stability of Geminin Complexes. Metaphase and interphase extracts fractionated by size exclusion chromatography both contain two geminin species. Peak 1 (blue boxes) and peak 2 (red boxes) of metaphase and interphase fractions were pooled and reapplied to the column for a second fractionation. Elutions were analysed by western blot for geminin.

Preliminary experiments were performed with metaphase extract on a broad glycerol gradient of 5-50% to observe the sedimentation of geminin and Cdt1 (Figure 45). The refractive index of fractions in a control run is shown to demonstrate that linear gradients had formed. Molecular weight markers were run with each experiment as there is experiment-experiment variation. A single geminin complex was observed which fractionated well with Cdt1. In an attempt to gain a greater resolution across geminin and Cdt1, samples metaphase and interphase extracts were applied to a 5-28% glycerol gradient. The refractive index and molecular weight marker controls are shown (Figure 46). Geminin and Cdt1 fractions were analysed by western blot, quantified by 2D densitometry and intensity was plotted against fraction number to demonstrate the distribution of proteins (Figure 47). Both geminin and Cdt1 eluted at smaller apparent molecular weights in interphase compared to metaphase extract, consistent with gel

filtration data, however the resolution of the gradient was not sufficient to separate geminin and Cdt1 in interphase extracts.

The distribution of molecular weight markers by gel filtration and glycerol gradient sedimentation was plotted to generate standard curves to estimate the Stoke radius and Sedimentation coefficient, respectively (Figure 48). These data were then used to calculate the molecular weight of geminin and Cdt1 containing complexes using the Siegel and Monty equation, $M = 420.5(R_sS)$ (Erickson, 2009).

The calculated molecular weight for the metaphase geminin-Cdt1 complex was 144 kDa (Figure 48). In a stoichiometric switch mechanism geminin-Cdt1 complexes are hypothesised to switch from a permissive heterotrimer to an inhibitory heterohexamer, in response to a cell cycle regulatable factor, such as local concentration or PTM. However, the molecular weight of the inhibitory complex, estimated by Siegel and Monty, does not fit well to a heterohexamer (geminin:Cdt1 at 4:2) which has an estimated molecular mass of 240 kDa, but does fit closer to a heterotrimer (geminin:Cdt1 at 2:1) which has an estimated molecular mass of 120 kDa. In fact the 144 kDa geminin-Cdt1 complex fits best to a tetramer (geminin:Cdt1 at 3:1) which has a molecular mass of 144 kDa, however it can be expected that geminin is a constitutive dimer in extracts. It has been suggested that the average error for molecular weight estimations by Siegel and Monty is $\pm 10\%$ (Erickson, 2009). Taking this into account a 2:1 heterotrimer is a much more likely fit. If the 2:4 (Cdt1:geminin) heterohexamer is indeed the inhibited form of Cdt1, it is not stable enough to be maintained during gel filtration. The Cdt1-independent portion of metaphase geminin, estimated to be 109 kDa, appears to form a tetramer ($4 \times 25 \text{ kDa} = 100 \text{ kDa}$). The larger interphase geminin complex, estimated to be 93 kDa, is also close to a tetramer but runs slightly smaller. This may represent a tetramer with an altered shape in interphase or metaphase extracts.

The smaller interphase geminin complex, estimated to be 73 kDa, which is similar to a trimer ($3 \times 25 \text{ kDa} = 75 \text{ kDa}$). This may represent a dimer with an altered shape. Alternatively interphase peak 1 and peak 2 may consist of dimers bound by interacting proteins, as these interphase geminin complexes were salt sensitive.

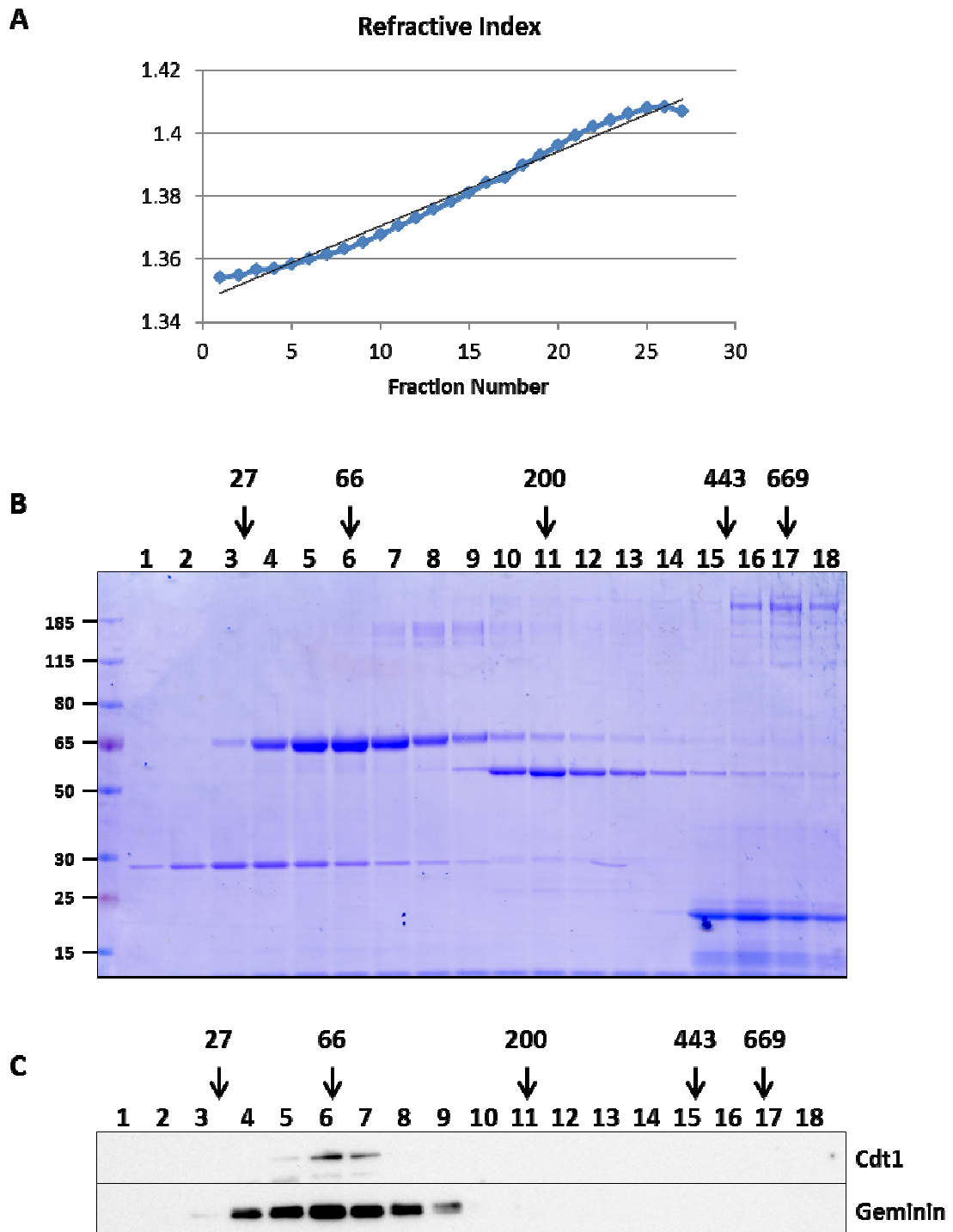


Figure 45: Sedimentation of Metaphase Extract Through a 5-50% Glycerol Gradient.(A) A control tube was run to demonstrate the linearity of the gradient. (B) Gel filtration markers or (C) 100 μ l of 1/5 diluted metaphase arrested extract were applied to a 5 ml 5-50% glycerol gradient and proteins were separated by centrifugation for 16 hours at 363,137 x g. 200 μ l fractions were collected and analysed by SDS-PAGE followed by Coomassie staining or western blot analysis for geminin and Cdt1.

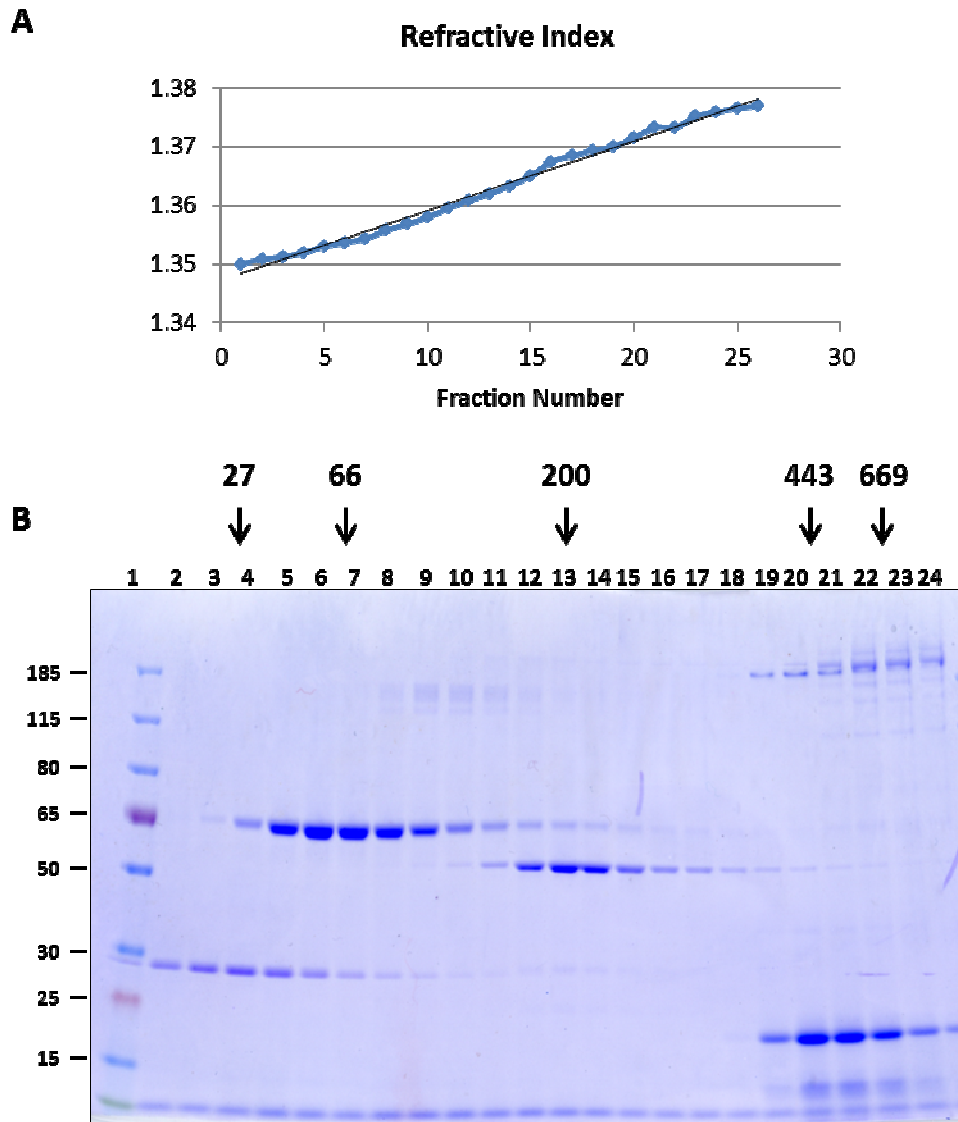


Figure 46: 5-28% Glycerol Gradient Controls.(A) A control tube was run to demonstrate the linearity of the gradient. **(B)** Gel filtration markers were applied to a 5 ml 5-50% glycerol gradient and proteins were separated by centrifugation for 16 hours at 363,137 x g. 200 μ l fractions were collected and analysed by SDS-PAGE followed by Coomassie staining. These controls were run alongside metaphase and interphase extract samples (Figure 47).

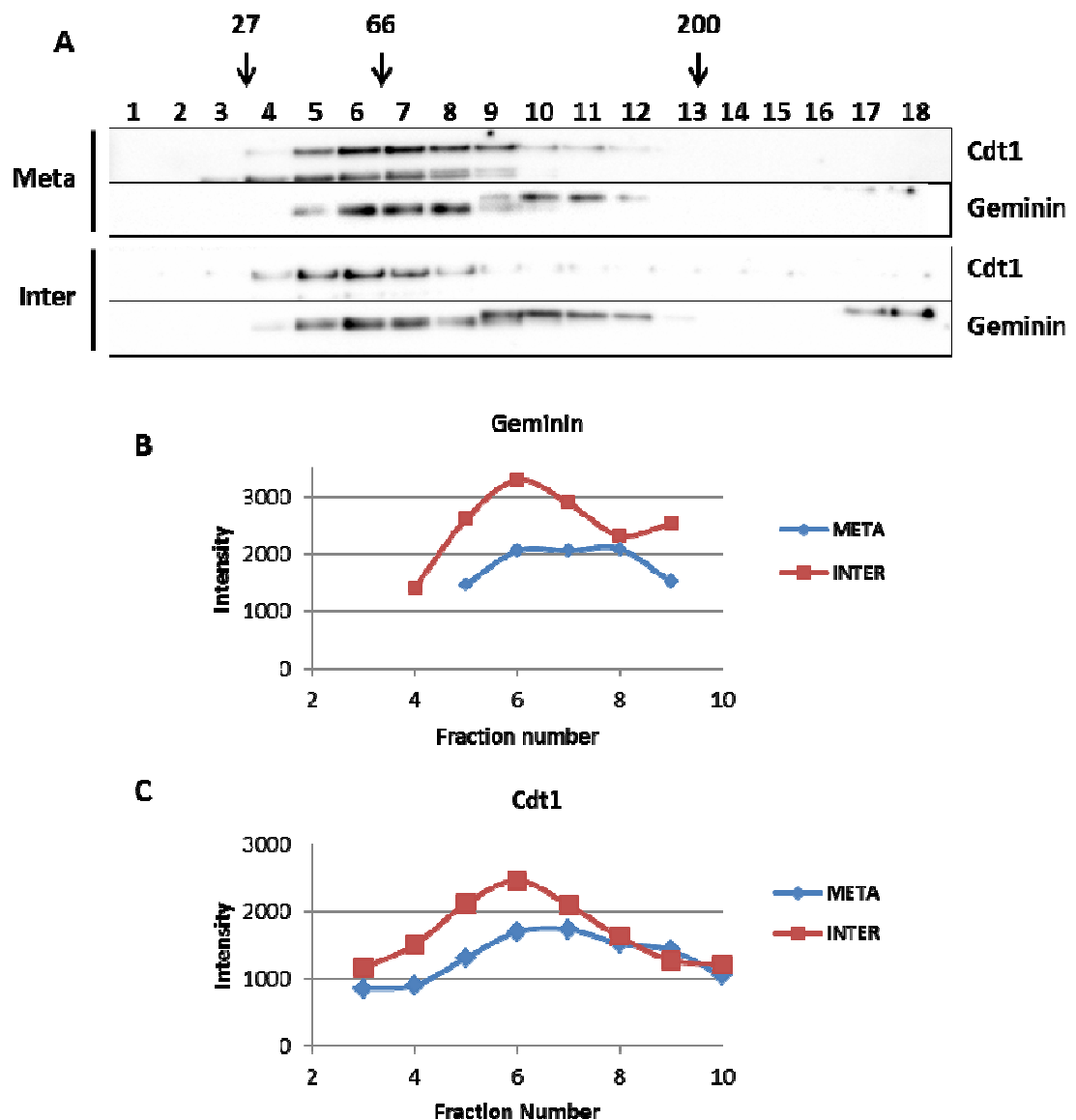


Figure 47: Sedimentation of Extracts Through a 5-28% Glycerol Gradient.(A) 100 μ l of 1/5 diluted metaphase and interphase extracts were applied to a 5 ml 5-28% glycerol gradient and proteins were separated by centrifugation for 16 hours at 363,137 x g. 200 μ l fractions were collected and analysed by western blot analysis for geminin and Cdt1.(B and C)The intensity of geminin and Cdt1 in metaphase and interphase fractions were quantified by 2D densitometry.

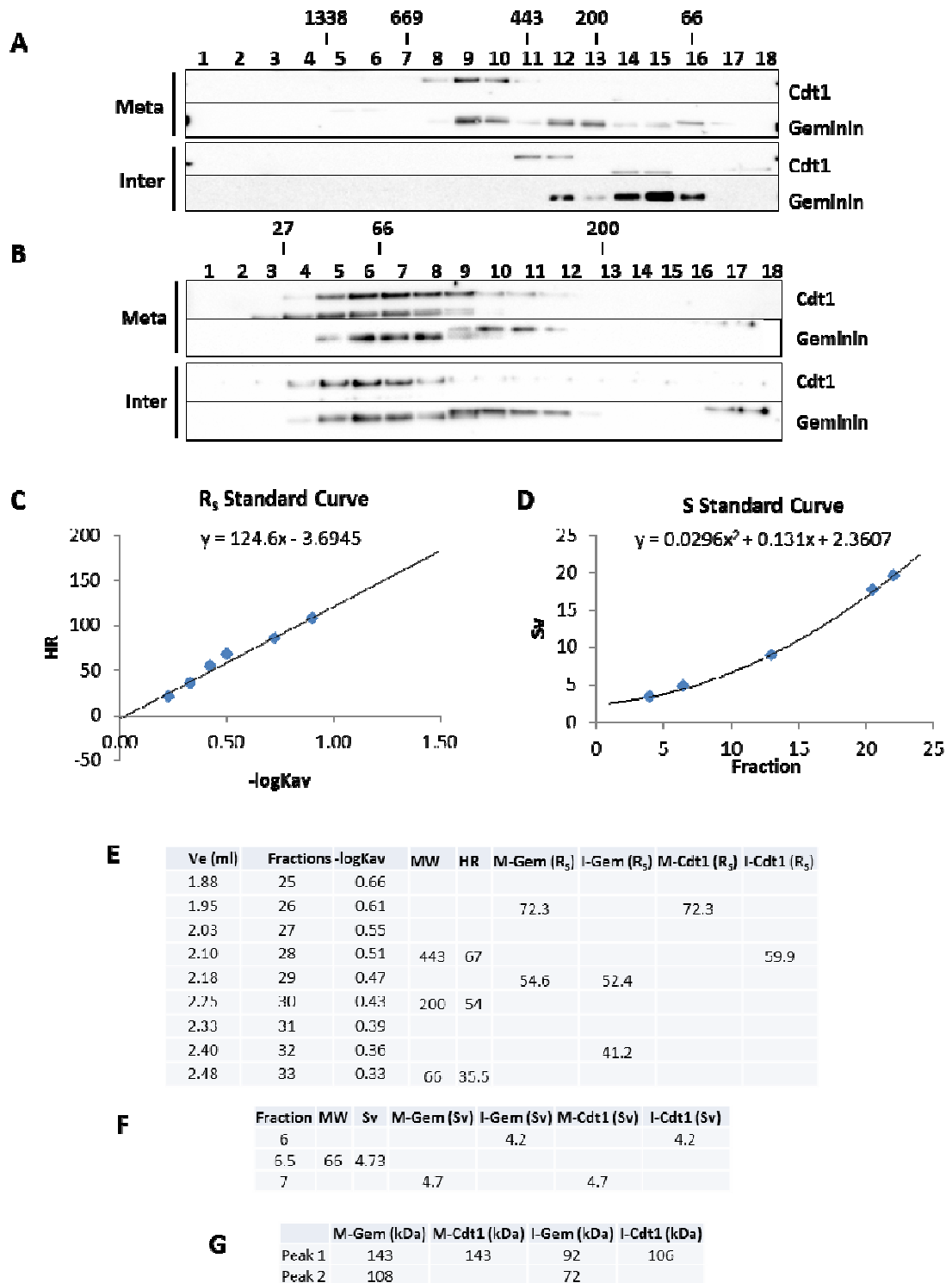


Figure 48: Molecular Weight Estimation by Siegel and Monty. (A) Fractionation of molecular weight markers and metaphase and interphase extracts by SEC. (B) Sedimentation of molecular weight markers and metaphase and interphase extracts on a 5-28% glycerol gradient. (C) Molecular weight markers from A were used to plot a standard curve for the Stokes radius (R_s). (D) Molecular weight markers from Figure 46 were used to plot a standard curve for the sedimentation coefficient (S_v). (E) R_s was calculated

for peak fractions in A using the standard curve C. (F) S_v was calculated for peaks in B using the standard curve D. (G) Molecular weights were calculated with the equation $M = 420.5(S.R_s)$.

5.4 Attempts to Reconstitute Geminin-Cdt1 Complexes with Recombinant Proteins

The molecular weights of endogenous complexes calculated by Siegel and Monty cannot all be accounted for simply by variations of geminin and Cdt1 proteins alone. This discrepancy between the estimated molecular weights and predicted molecular weights of complexes with specific stoichiometries allows for speculation on the role of novel interacting proteins in egg extracts. To gain further insight into the makeup of geminin and Cdt1 complexes and determine whether observed complexes consist of geminin and Cdt1 alone or may involve additional proteins, it would be informative to reconstitute complexes with recombinant proteins for analysis by size exclusion chromatography. Any geminin or Cdt1 containing complexes observed in extracts that do not correlate well to recombinant complexes would likely contain an interacting protein, or are folded into an altered shape.

Expression of wild-type His₆-Geminin in bacteria was found to produce mostly insoluble protein and any purified soluble protein would consistently smear on gel filtration, suggesting that it was unfolded. The geminin expression plasmid (which was used in the original geminin paper of McGarry and Kirschner (McGarry and Kirschner, 1998)) was sequenced and found to contain an extra 84 nucleotides inserted at the N-terminus between the thrombin cleavage site and the geminin start methionine (Figure 49). A BLAST search of the fragment found that it was made up of a multiple cloning site from a previous plasmid and a portion of geminin mRNA. Full length wild type geminin was cloned into a pET15b expression vector and protein expression was optimised to produce high amounts of soluble protein. This was achieved by reducing the incubation temperature to 25°C after induction with IPTG (Figure 50).

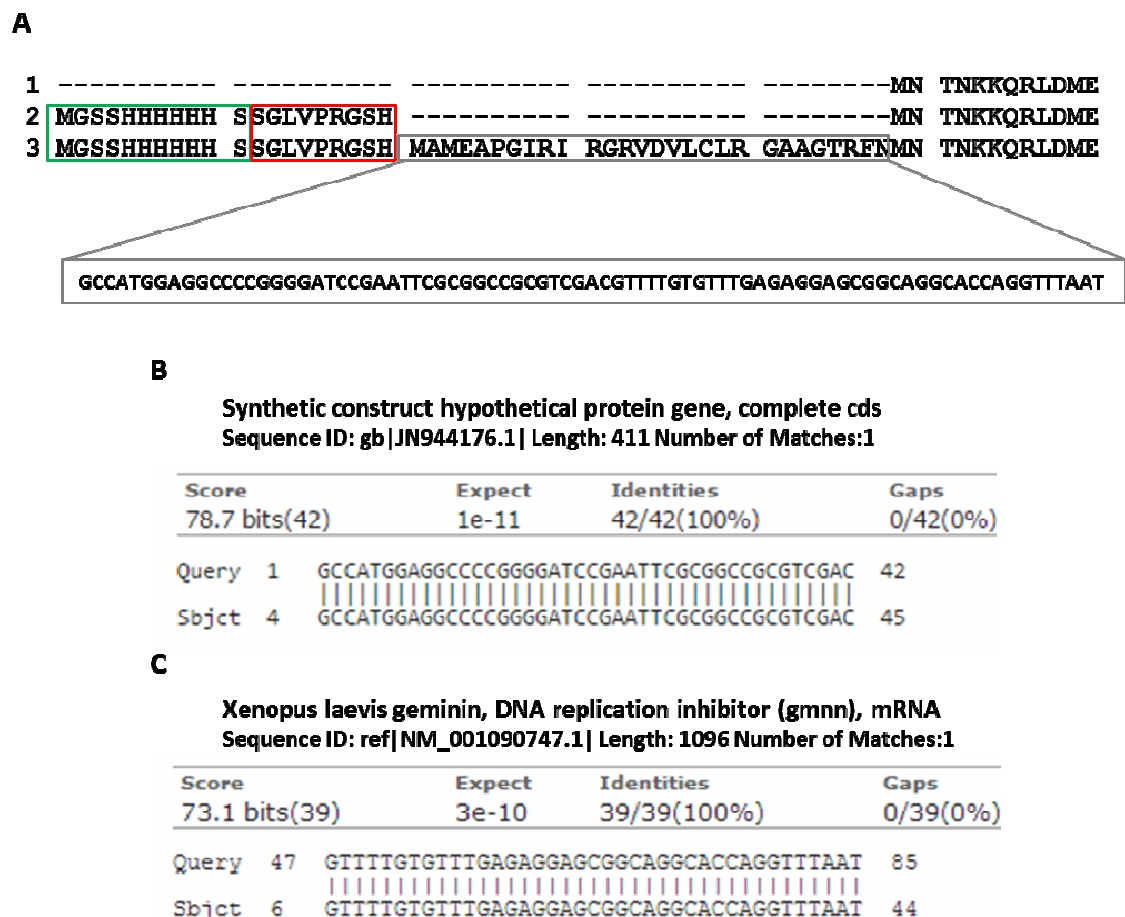


Figure 49: Recombinant Geminin Insertion.(A)Multiple sequence alignment of geminin (1), the geminin^{DEL} plasmid (2) and the wild type geminin plasmid (3). The green box denotes the His₆ tag. The red box denotes the Thrombin cleavage site. The black box surrounds the aberrant insertion. The nucleotide sequence was BLASTED and found to consist of a cloning plasmid multiple cloning sequence (B) and a portion of geminin mRNA (C).

As a control to show that this protein was active and therefore likely to be correctly folded, recombinant geminin was tested for its ability to inhibit DNA replication. Recombinant full length geminin or geminin^{DEL} was titrated into interphase extract 5 minutes prior to DNA addition and the levels of DNA replication were measured after 90 minutes (Figure 51). DEL is a more inhibitory protein, consistent with previous reports, coordinating a switch in licensing inactivation from 30-60 nM. Wild type protein fully inhibited licensing in a switch like manner from 120-240 nM, showing that this protein was active and therefore was likely to be correctly folded.

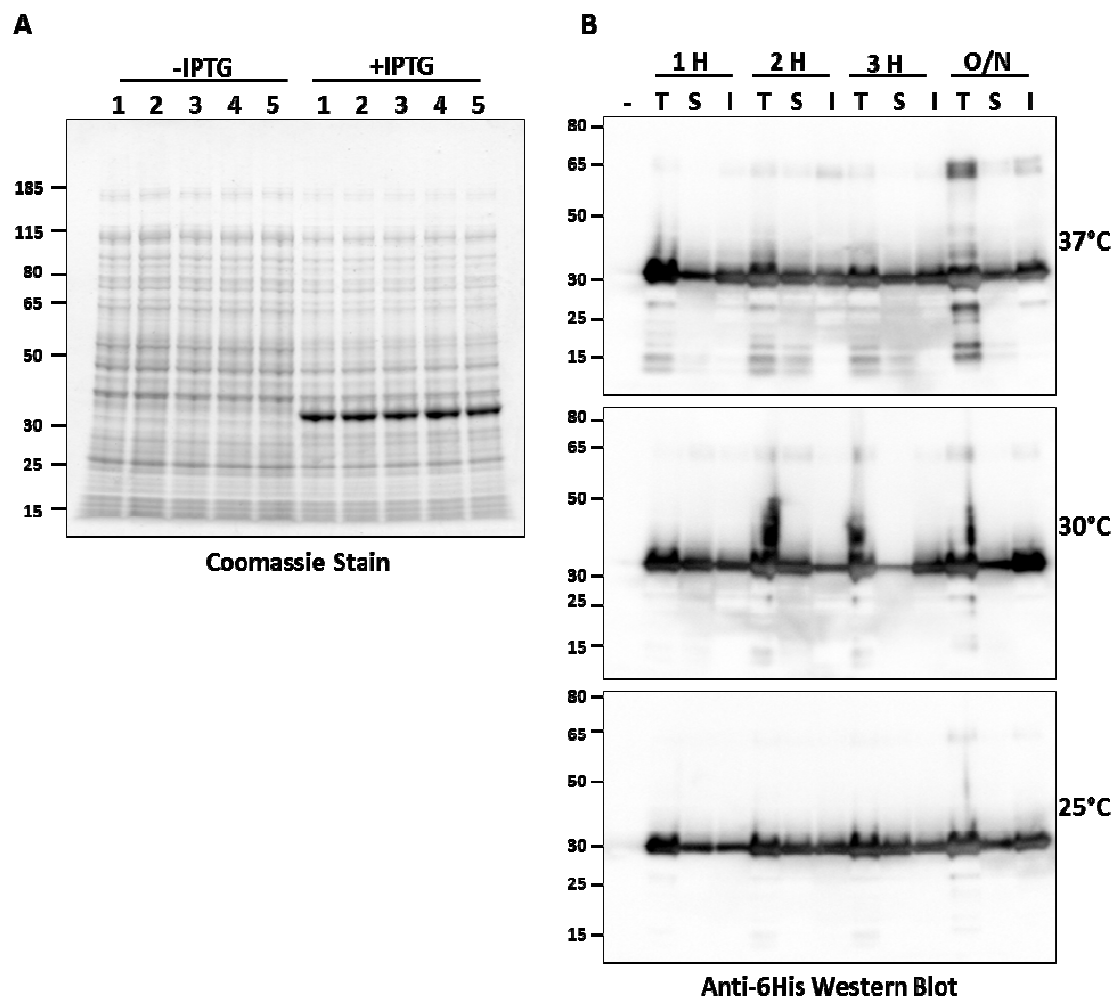


Figure 50: Optimisation of Soluble Geminin Expression. (A) 5 clones were selected and assessed for their ability to express geminin after stimulation with IPTG. (B) The time and temperature of expression was optimised to produce high levels of soluble protein. Expression for 3 hours at 25°C produced high levels of soluble protein (T: total, S: soluble, I insoluble).

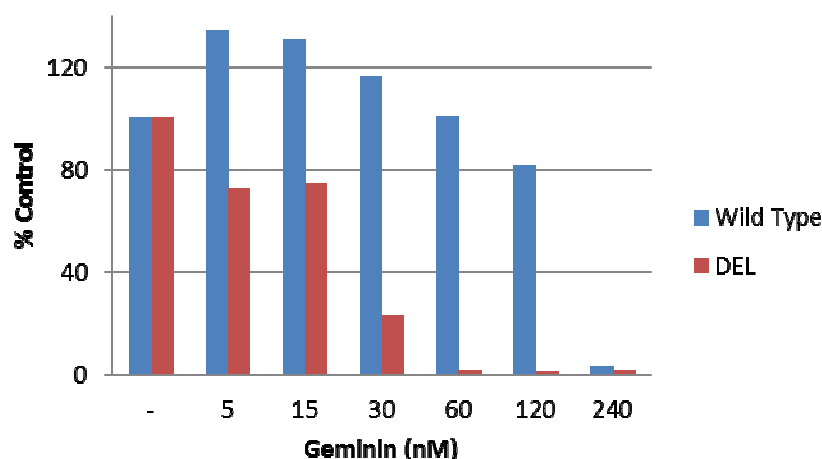


Figure 51: The Activity of Full Length Recombinant Geminin. Extract released into interphase was supplemented with a titration of geminin^{DEL} or wild type His₆-Geminin prior to DNA addition. DNA replication measured after 90 minutes and expressed as a percentage of the no addition control.

To determine whether this active recombinant protein alone can form complexes resembling those observed in extracts, recombinant geminin was fractionated by size exclusion chromatography (Figure 52). The peak fractions eluted between 443 and 200 kDa, resembling the second geminin peak of metaphase arrested extract, which has been previously shown consists of active geminin. Therefore the second peak in metaphase is likely to exist as a geminin-only complex. At an estimated molecular weight of 108 kDa, this complex of endogenous geminin that is able to bind Cdt1 and inhibit licensing is likely to be a tetramer, or dimer of dimers. Therefore the recombinant protein alone in solution is also assumed to be a tetramer. Interestingly, geminin complexes 1 and 2 from interphase extracts are estimated to have slightly smaller molecular weights, suggesting they may not be capable of tetramer formation. Interphase geminin complexes were sensitive to high salt. Therefore, in an attempt to break the recombinant tetramer and observe the elution profile of a geminin dimer, the recombinant protein was fractionated by size exclusion chromatography in the presence of increasing salt concentrations. A broadening of the geminin peak was observed in the presence of 500 mM NaCl. At 1000 mM the peak elution shifted a fraction to the right, eluting at 200 kDa. The broadening of the peak at 500 mM salt suggests that there is a dynamic

interaction of geminin dimers to form tetramers and this interaction is disrupted by high salt. Assuming the recombinant geminin is a tetramer at low salt suggests that in high salt a dimer is formed at 200 kDa. Therefore geminin dimers in extract would be expected to fractionate at 200 kDa. Interphase geminin complexes fractionate to either side of this, suggesting geminin dimers are not present in extract or that they are modified in some way that alters their hydrodynamic radius. This hints that interphase geminin is either bound by an interacting protein, or has an altered shape.

To get a more conclusive measure for the molecular weight and in turn stoichiometry of recombinant geminin complexes, recombinant geminin was analysed by SEC-MALS (Size Exclusion Chromatography with Multi-Angle Light Scattering analysis). In a properly calibrated system the light scattering can be used to accurately measure the molecular mass of a complex. The recombinant protein shown in Figure 52 was purified by an optimised protocol which used LFB100 buffer. During initial SEC-MALS experiments proteins were applied to a Mab-PAC SEC-1 column in the presence of LFB100 buffer, however issues surfaced with proteins eluting much later than expected, suggesting that at the high concentrations required for SEC-MALS the protein was interacting with the matrix of the column. The buffer was simplified (40 mM HEPES, 100 mM KCl, pH 8.0) and recombinant protein was run on a 24 ml Superdex 200 column. An initial experiment was undertaken under these conditions with a relatively impure sample of recombinant geminin (Figure 53). The geminin specific peak gave a molecular weight of 120 kDa. This is consistent with a tetramer of recombinant geminin ($27.5 \text{ kDa} \times 4 = 110 \text{ kDa}$). There was a contaminating protein present in the sample which is likely to have increased the molecular weight slightly from ~ 110 to ~ 120 kDa. The specificity of the peak and the protein is shown. A duplicate sample was fractionated and elutions were collected and subjected to SDS-PAGE followed by

Coomassie staining or western blotting for His₆-tag. SEC-MALS shows that recombinant geminin which is able to inhibit licensing consists of a tetramer.

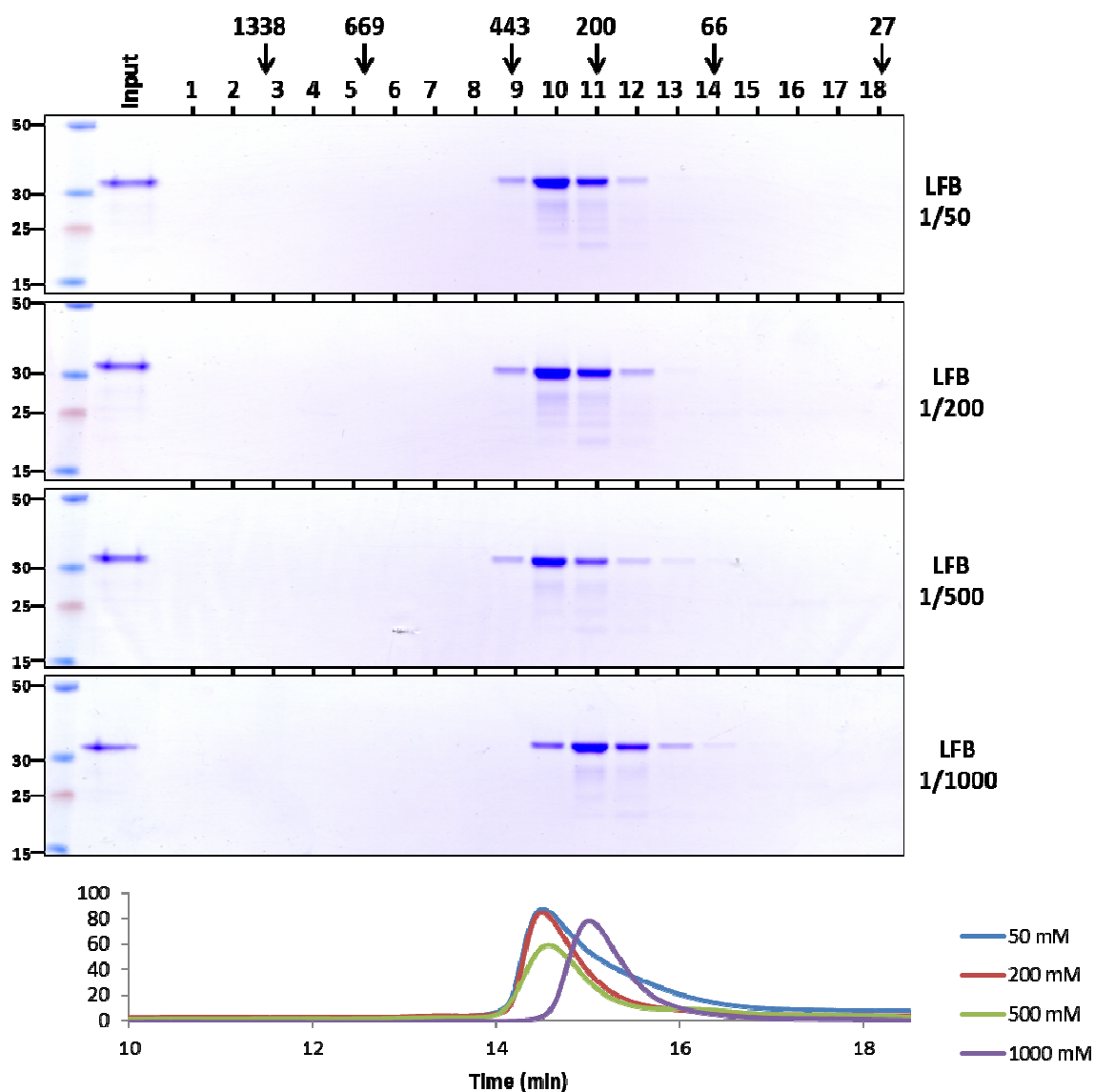


Figure 52: Fractionation of Recombinant Wild-type Geminin. Full length wild-type His₆-Geminin was fractionated by size exclusion chromatography on a Mab-PAC SEC-1 column and fractions were analysed by SDS-PAGE and Coomassie staining. The UV trace is shown to demonstrate the broadening of the peak at higher salt concentrations.

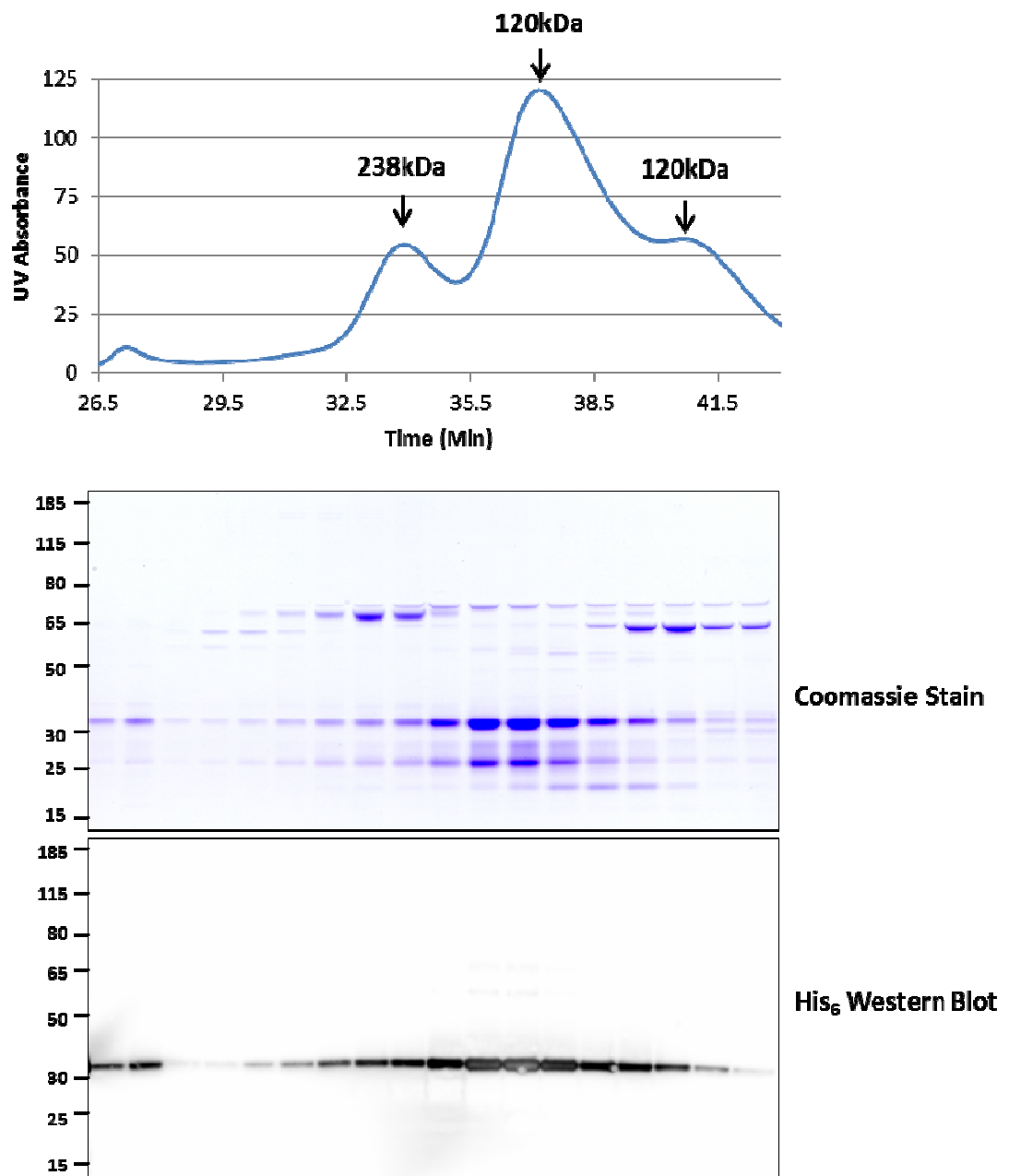


Figure 53: Recombinant Geminin Analysed by SEC-MALS is 120 kDa. The UV trace from the SEC-MALS experiment is shown. The geminin complex eluted in a peak at 37 minutes. A duplicate sample was fractionated by SEC only to demonstrate the specificity of geminin bands.

Next, attempts were made to reconstitute geminin-Cdt1 complexes. Recombinant Cdt1 was purified from insoluble pellets in bacteria under denaturing conditions and refolded in a THED200 buffer system. Cdt1 was liable to precipitate during the refolding of dialysis, and it appeared that the solubility depended not only on the concentration of Cdt1 but also on the concentration of contaminating proteins; the lower the Cdt1 concentration and the higher the amount of impurities the more soluble recombinant Cdt1 was. This made it difficult to analyse recombinant Cdt1 alone by chromatography as it was usually purified at a relatively low concentration and would readily precipitate on the columns, presumably once the separated from the stabilising contaminating proteins.

Experiments were designed to vary the ratios of geminin and Cdt1 and to analyse complex formation by size exclusion chromatography at various salt concentrations, but this was not achieved. Recombinant Cdt1 only stayed in solution at very high concentrations of recombinant geminin on a MAbPac SEC-1. This was consistent with previous work undertaken in the lab that showed that Cdt1 required either IGG, BSA or PVA to stay in solution during chromatography ((Tada et al., 2001)). The complex of geminin alone peaked between 443 and 200 kDa. Preincubation of His₆-geminin with His₆-Cdt1 for 15 minutes prior to size exclusion chromatography resulted in a shift of a small portion of geminin to a smaller elution volume suggesting that these proteins had formed a complex (Figure 54). This reconstituted geminin-Cdt1 complex eluted at 443 kDa, much lower than expected compared to geminin-Cdt1 inhibitory complexes found in metaphase extract which elute closer to 669 kDa. This recombinant complex appears more similar to interphase Cdt1 which also elutes at 443 kDa, suggesting that His₆-geminin and His₆-Cdt1 may not interact in a similar manner to the interactions between endogenous proteins.

To ensure the issues with Cdt1 stability were not column-specific, additional chromatography was performed on a Superose 6 column with PVA containing buffer. Initial experiments under these conditions did seem to maintain Cdt1 stability and allowed for a more stoichiometric reconstitution of geminin-Cdt1 complexes, however all complexes ran at 443 kDa and did not appear to resemble the inhibitory geminin-Cdt1 complexes identified in extract.

5.5 Is There a Dynamic Stoichiometric Switch?

Chromatin becomes maximally licensed approximately 15 minutes after the addition of calcium to metaphase arrested extracts (Blow, 1993). The striking difference in the kinetics of geminin and Cdt1 destruction that was observed (Figure 8 and Figure 9) suggested that the activity of the APC/C creates a period within 10 minutes where the ratio of geminin to Cdt1 is dramatically altered. While geminin is reduced to approximately 40-50% at this timepoint, Cdt1 levels are relatively stable. Therefore the relative levels of geminin and Cdt1 change during the licensing period, which has been hypothesised to control the stoichiometry and in turn the activity of these proteins. It has also been shown that the ability to license chromatin falls over time after the addition of calcium to metaphase extracts (Mahbubani et al., 1997). This loss of activity could be explained by the slow change in the relative levels of these proteins due to the linear reduction of Cdt1. As geminin levels stabilise and Cdt1 destruction begins after approximately 20 minutes the relative levels would change to favour licensing inhibition. A simple way to investigate this stoichiometric switch hypothesis would be to determine the relative ratio of geminin to Cdt1 after the metaphase-anaphase transition and throughout the licensing period. For this, an accurate quantification of endogenous proteins in metaphase arrested extracts was required. With a known amount of protein in metaphase arrested extracts, the molar ratio of proteins can be plotted over time using the destruction timecourse data.

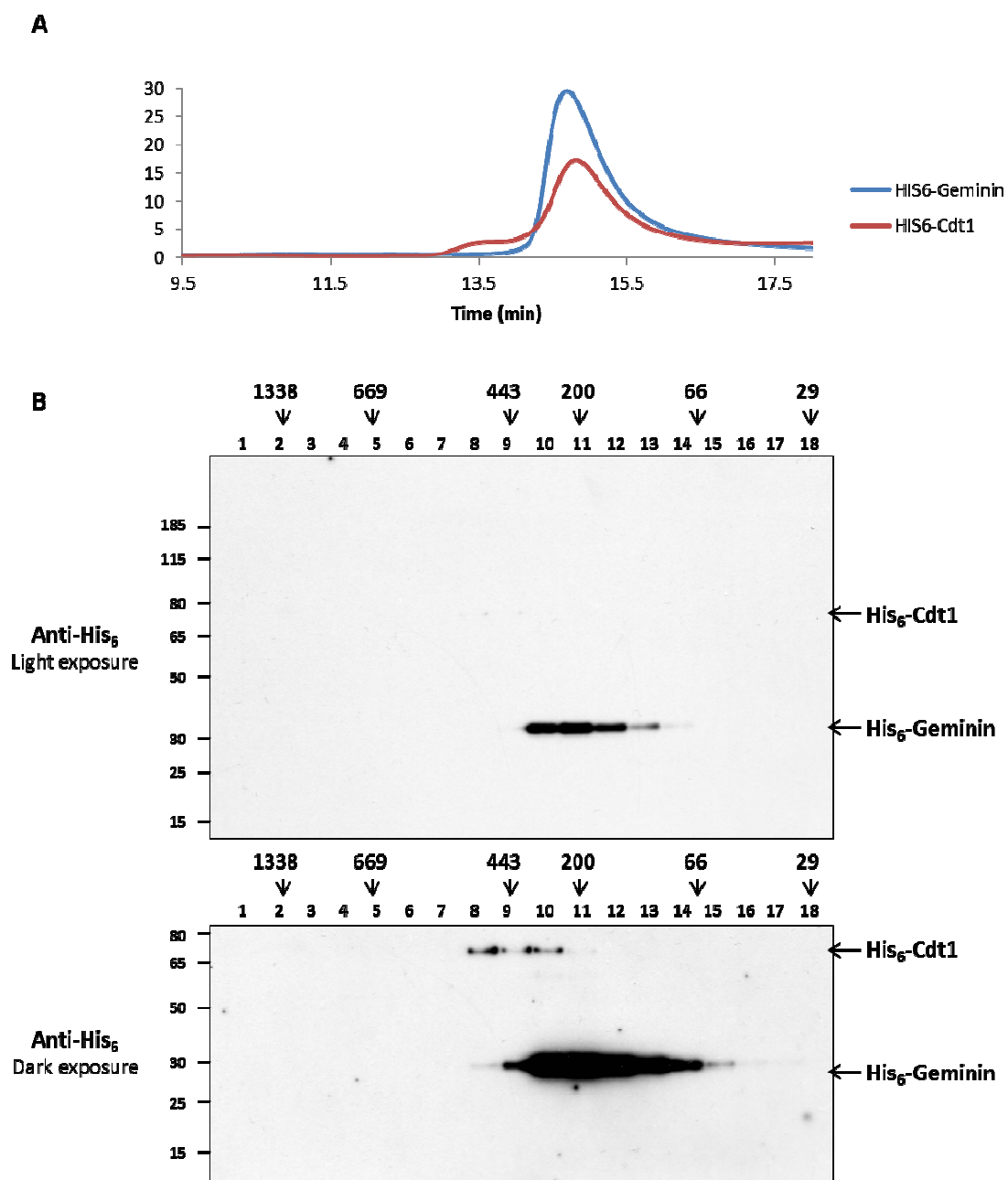


Figure 54: Formation of Recombinant Geminin-Cdt1 Complexes. (A) UV absorption of recombinant complexes fractionated on a MAbPac SEC-1. (B) Fractions were analysed by western blot analysis for His₆ to detect both geminin and Cdt1. A dark exposure is shown to demonstrate the Cdt1 peak. In addition this exposure emphasises the relative ratios required to maintain Cdt1 in solution.

Recombinant full-length Cdt1 and geminin were quantified against BSA standards of known concentrations (Figure 55). Recombinant proteins of known concentrations were then used to quantify endogenous protein concentrations by western blot and densitometry analysis. The concentration of geminin and Cdt1 in metaphase extracts was found to be 55 and 41 nM, respectively.

Experimental data was taken from Figure 9 to calculate the changes in the molarity of geminin and Cdt1 over time after the addition of calcium. Molar ratios were then calculated and plotted over time (Figure 56). Both the ratio of geminin to Cdt1 (G/C) and the ratio of Cdt1 of geminin (C/G) are shown. Although they are essentially the same data, plotting of both together gives a greater appreciation of the switches in ratios that were observed. In the absence of DNA the relative amount of Cdt1 increases rapidly as geminin is degraded in response to calcium addition (Figure 56A). An immediate switch is observed and Cdt1 becomes more concentrated than geminin by 4 minutes. The relative concentration of Cdt1 continues to rise until a peak is observed at 16 minutes. This correlates well with previous reports that demonstrated that the licensing system becomes fully activated 15 minutes after calcium addition (Blow, 1993). Cdt1 remains relatively more concentrated than geminin until 60 minutes after calcium addition, after which time geminin becomes relatively more concentrated due to APC/C-mediated destruction of Cdt1. This is again consistent with previous reports which stated that licensing factor activity is lost from 60-120 minutes due to the loss of Cdt1 activity (Mahbubani et al., 1997). These data fit very well to the hypothesis that the relative ratios of geminin to Cdt1 can coordinate the Cdt1-dependent licensing activity. In the presence of 3 ng/ μ l of DNA the switches in the relative ratios are even more striking (Figure 56B). The addition of DNA has no effect on the initial degradation of geminin and an immediate switch is observed with Cdt1 becoming more

concentrated at 4 minutes and reaching maximal levels at 15 minutes. Within the next 30 minutes nuclear formation occurs leading to the initiation of DNA replication. DNA replication dependent destruction of Cdt1 then leads to a pronounced switch in the relative concentration of geminin at 45 minutes. If activity is regulated by the relative ratios of these proteins then the observed data for extracts supplemented with 3 ng/ μ l DNA would suggest that Cdt1 remains active from 15-45 minutes. If this was the case then rereplication is likely to occur. As soon as nuclear envelopes are assembled, which usually takes 10-25 minutes, geminin nuclear import is likely to increase the relative ratio of geminin to Cdt1. Therefore the relative ratios of geminin to Cdt1 are likely to switch much sooner than 45 minutes within individual nuclei. Taken together this data is consistent with a mechanism where the activity of replication licensing is influenced by the relative ratios of geminin and Cdt1.

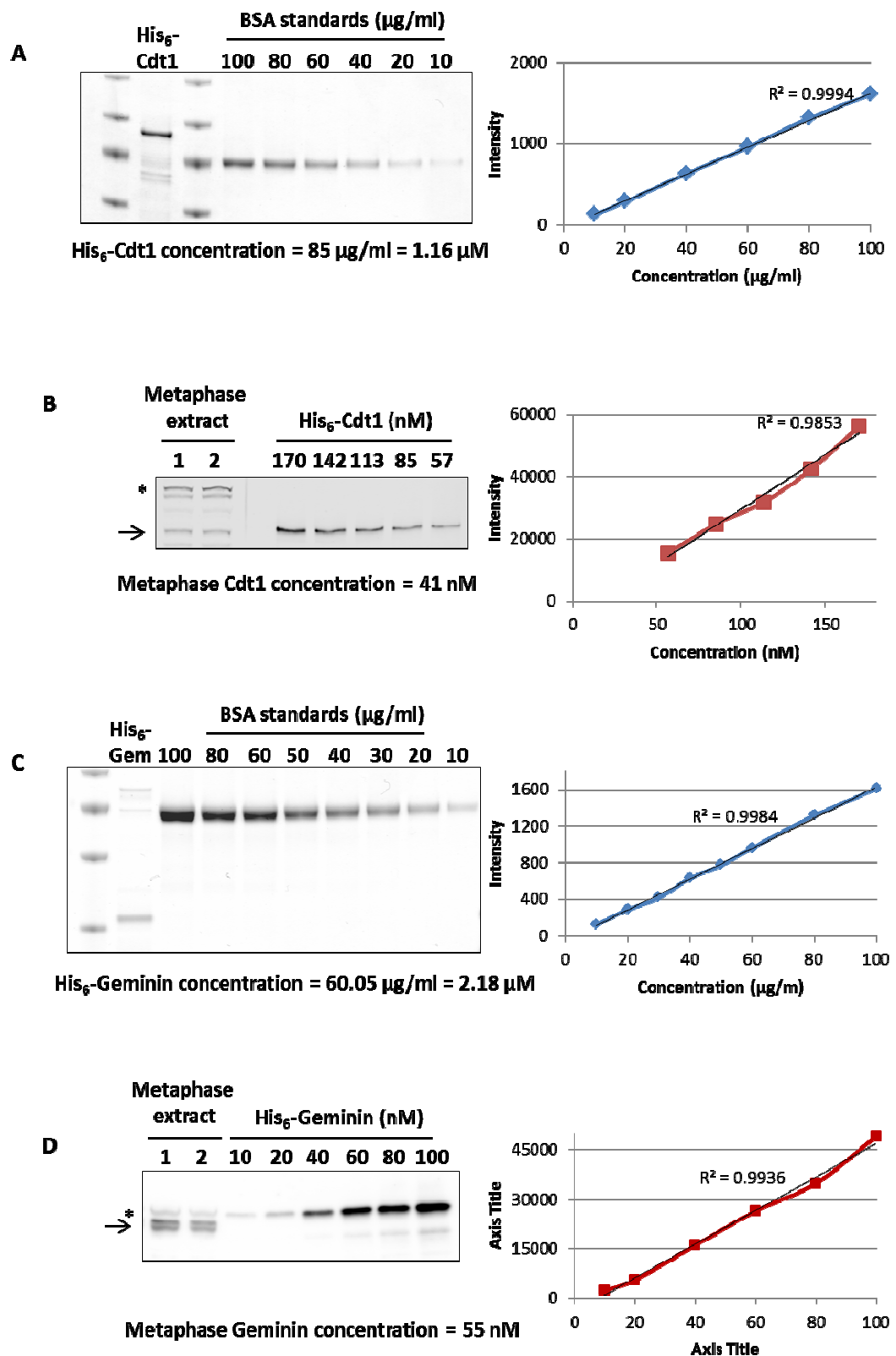


Figure 55: Quantification of Endogenous Geminin and Cdt1 in Metaphase extract. (A) Recombinant Cdt1 was quantified against a BSA standard of known concentration. (B) Cdt1 quantified in A was used to determine the concentration of protein in metaphase extract. (C) Recombinant geminin was quantified against a BSA standard of known concentration. (D) Geminin quantified in C was used to determine the concentration of protein in metaphase extract.

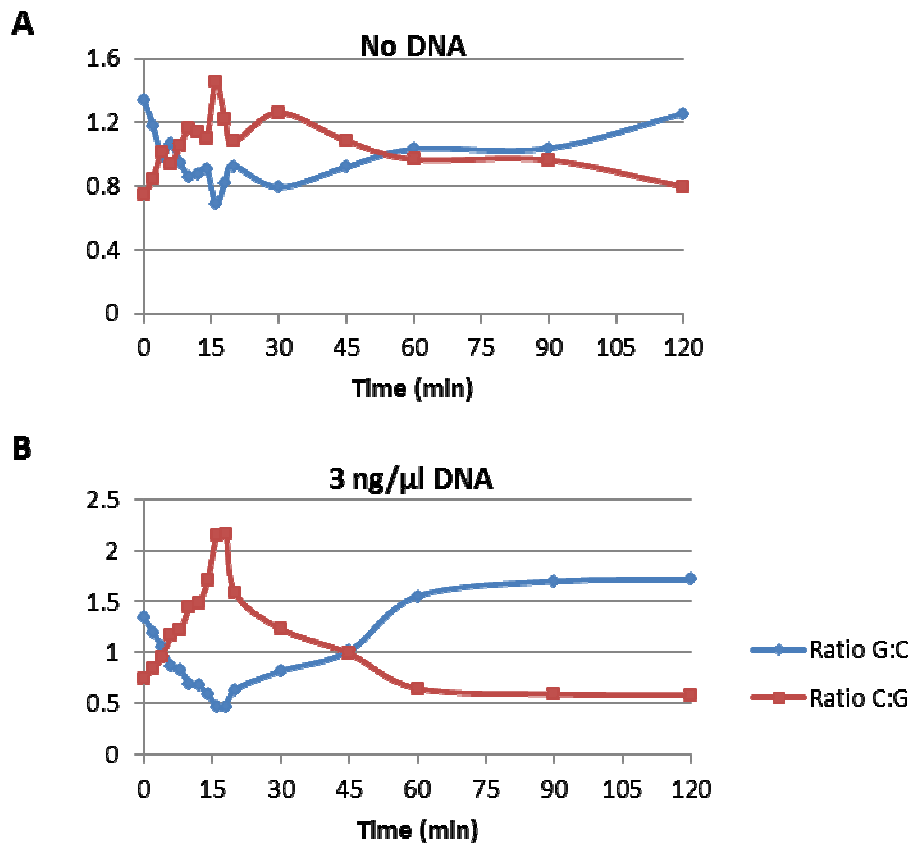


Figure 56: The Change in Molar Ratio of Geminin and Cdt1 Complexes Over Time. Data from Figure 10 and Figure 55 was used to determine the change concentrations of geminin and Cdt1 during timecourses. This was used to calculate the Molar ratio of geminin to Cdt1 (and vice versa) and plotted over time. While both curves are essentially the same data both were plotted to better appreciate the observed switches in relative ratios over time. **(A)** Changes in the relative ratios of geminin and Cdt1 over time in extract. **(B)** Changes in the relative ratios of geminin and Cdt1 over time in extract supplemented with 3 ng/μl of DNA.

A dynamic stoichiometric switch hypothesis has been proposed (De Marco et al., 2009). There is good evidence here that there is an on/off switch in the ability to license DNA which depends on the relative concentrations of geminin and Cdt1. The next step was to determine how this concentration dependent change was implemented and whether it is mediated by complex assembly as hypothesised. Therefore it was of interest to prove the existence of two different geminin:Cdt1 complexes. So far we had not observed complexes larger than the one at 669 kDa and no evidence that the samples of geminin and Cdt1 that cofractionate in interphasal were interacting or not. So to determine if

there are two geminin-Cdt1 complexes with differing stoichiometries geminin samples were immunoprecipitated from SEC fractions. Geminin was immunoprecipitated across 11 fractions and analysed for the ability to pull down Cdt1 (Figure 57). The supernatants were run after the IP to show the levels of protein depletions. Geminin immunoprecipitated from metaphase gel filtration samples was present in two peaks as expected, with the larger peak efficiently pulling down endogenous Cdt1 (Figure 57A). In the supernatant, both geminin and Cdt1 were depleted. Geminin immunoprecipitated from interphase extract produced two peaks as expected (Figure 57B). Fractions 5 and 6 were predicted to form an interphase geminin-Cdt1 complex which would be permissive for licensing. However no Cdt1 was co-immunoprecipitated with geminin in these fractions and supernatant are shown to contain Cdt1 while geminin samples were sufficiently depleted. This data suggests that interphase geminin does not form any complexes with Cdt1. This is consistent with earlier experiments that showed Cdt1 addition to interphase extract did not result in an alteration of geminin fractionation (Figure 16). Taken together this data suggests that although the relative concentrations of geminin and Cdt1 may play a role in the activation of licensing this role is not mediated through a dynamic formation of geminin-Cdt1 complexes of different stoichiometries.

5.6 The Geminin Family of Proteins

The identification of two proteins with geminin-like coiled-coils, Idas and GemC1, defined a new family of geminin-like proteins (Balestrini et al., 2010, Pefani et al., 2011). The geminin coiled-coil mediates dimerisation and this is required for the inhibition of licensing. Due to the sequence similarity of the coiled-coil regions these proteins may be able to interact with geminin and therefore have good potential to represent novel geminin inhibitor proteins.

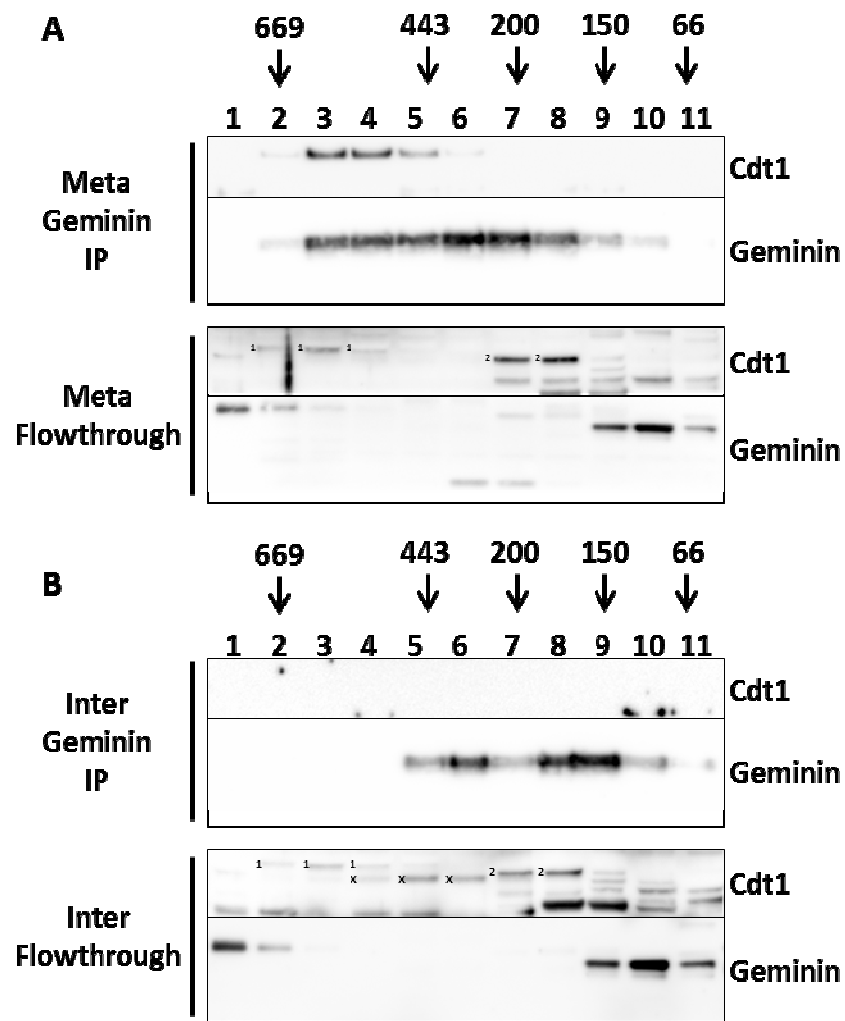


Figure 57: Immunoprecipitation of Geminin from SEC fractions. (A) Metaphaseextract was fractionated by size exclusion chromatography and geminin was immunoprecipitated from 11 fractions and analysed for the ability to pull down Cdt1. The supernatants were run after the IP to show the levels of protein depletions.(B) Interphaseextract was fractionated by size exclusion chromatography and geminin was immunoprecipitated from 11 fractions and analysed for the ability to pull down Cdt1. The supernatants were run after the IP to show the levels of protein depletions. 1 and 2 mark non-specific bands. Cdt1 is marked with an X.

5.6.1 Idas is not present in *Xenopus* Egg Extracts

During the initial stages of this project a number of Idas antibodies were raised against 100 amino acid peptides from the N- and C- terminus (by Peter Gillespie) or full length Idas recombinant protein. Four antibodies were raised in total and each was affinity purified against its cognate antigen. However no bands were consistent among proteins bands by western blot and mass spectrometry analysis of immunoprecipitation samples failed to identify Idas in *Xenopus* egg extracts. During this project Idas was found to be a key regulator of multicilliate cell differentiation during organogenesis and it could coordinate cell cycle exit, centriole assembly and control the transcription of FoxJ1 in these cells (Stubbs et al., 2012). Here, it was shown that Idas expression was first detected during gastrulation. Gastrulation occurs in stage 12 embryos and therefore Idas should be absent from present in *Xenopus* egg extracts, as appeared likely from the lack of specific bands.

5.6.2 GemC1 is a Geminin Interacting Protein

GemC1 was proposed to play a role in the initiation of DNA replication, specifically in the recruitment of Cdc45 to aid in CMG formation (Balestrini et al., 2010). This study showed that GemC1 does not bind to Cdt1 but the authors did not investigate the interaction of GemC1 with geminin. GemC1 antibodies were raised against an N-terminal 1-97 peptide (Amino acids 1-97, by Peter Gillespie) and full-length recombinant His₆-GemC1. A specific band could commonly identified by each antibodies was not found (Figure 58). However, the ability of each antibody to recognise GemC1 was demonstrated by their ability to recognise MBP-GemC1 by western blot (Figure 58). Affinity purification of antibodies was carried out and attempts were made to immunoprecipitate GemC1 and identify a specific band. Antibody light chains migrate to the molecular weight predicted for GemC1 by SDS-

PAGE, making band identification impossible by immunoprecipitation and western blot analysis. IPs using crosslinked antibodies may have negatively affected the antibodies as no bands were identified by SDS-PAGE and western blotting. However, in an attempt to identify GemC1, mass spectrometry analysis was undertaken on a gel slice between cut between 30-50 kDa from a non-crosslinked GemC1 IP. This resulted in the identification of a just a single GemC1 peptide with a low abundance. Taken together this demonstrates that multiple antibodies were generated which were capable of detecting GemC1, however it is likely that GemC1 was present at a low concentration in *Xenopus* egg extracts.

To determine whether GemC1 can interact with geminin MBP-GemC1 was incubated in metaphase and interphase extracts and pulled down with amylose resin. Samples were immunoblotted for MBP-GemC1 with a GemC1 antibody, a proposed interacting protein, TopBP1, and for geminin. MBP-GemC1 was found to specifically pull down a significant proportion of geminin in both metaphase and interphase extracts and no geminin was pulled-down in the absence of MBP-GemC1 (Figure 59A). TopBP1, however, was not detected suggesting that these proteins do not interact in extracts. This was unexpected as TopBP1 hypothesised to recruit GemC1 and in turn Cdc45 for the initiation of DNA replication (Balestrini et al., 2010). Therefore pulldowns were repeated and the interaction of MBP-GemC1 with TopBP1 and Cdc45 was assessed (Figure 59B). This was consistent with initial pulldowns, demonstrating that MBP-GemC1 and TopBP1 did not interact in metaphase or interphase extracts. MBP-GemC1 also failed to pulldown Cdc45 from metaphase and interphase extracts suggesting that these do not interact. In a reciprocal experiment TopBP1 was immunoprecipitated and samples were assayed for the co-precipitation of Cdc45 and GemC1 (Figure 59C). A significant amount of TopBP1 was specifically pulled-down with the anti-TopBP1 antibody in both metaphase and interphase extract, however no GemC1 or Cdc45 were

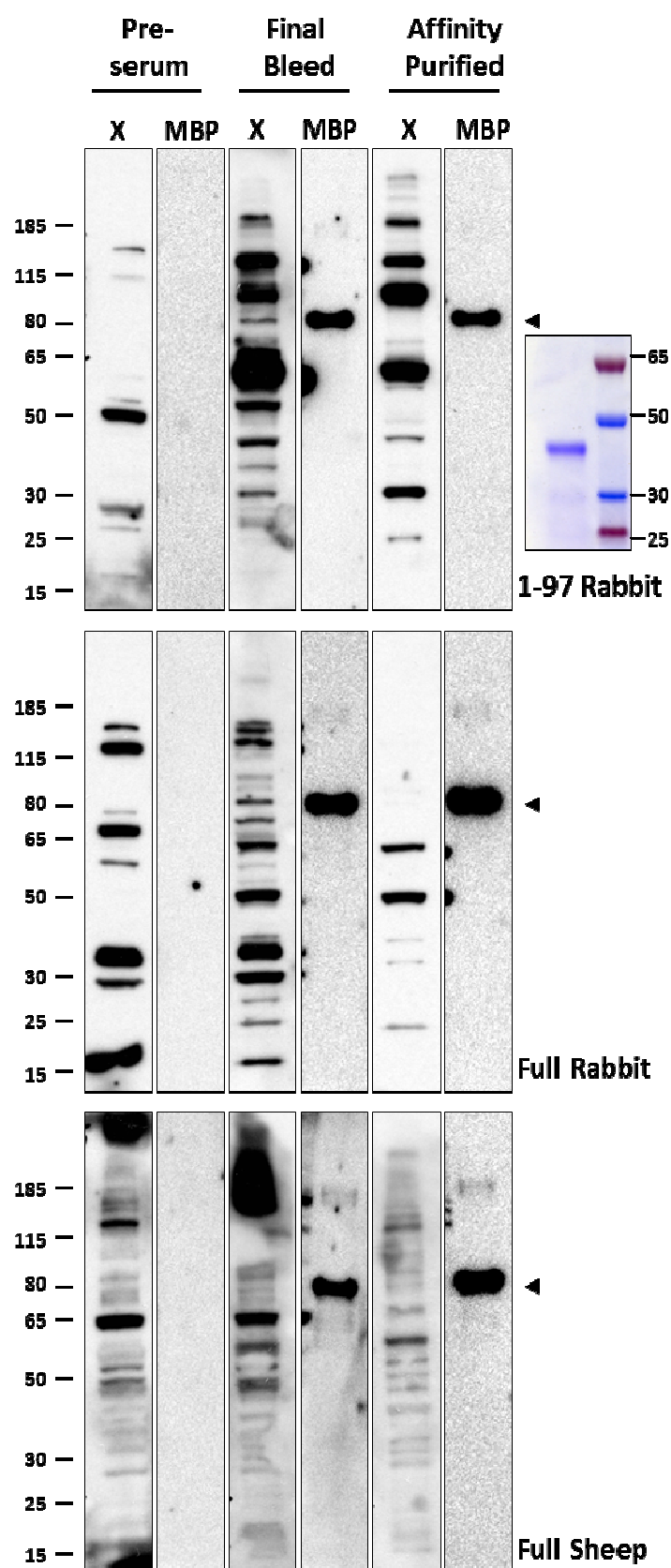


Figure 58: GemC1 Antibody Characterisation. In an attempt to characterise antibodies and identify a GemC1 whole extract (X) and MBP-GemC1 were separated by SDS-PAGE and western blotted with pre-immune serum, terminal bleed serum and affinity purified antibodies. A Coomassie stain of His₆-GemC1 separated by SDS-PAGE is shown to demonstrate the approximate size of the protein.

bound, suggesting that these proteins do not interact in the cytoplasm of metaphase and interphase extract.

GemC1 was first identified due to its sequence similarity to the geminin coiled-coil (Figure 60A). Geminin dimerization is mediated by the coiled-coil and the primary Cdt1 interaction site is located within the coiled-coil region. This suggests that GemC1 could interact with geminin via the coiled-coil domains and inactivate geminin by preventing the interaction with Cdt1. Having established that geminin could interact specifically with recombinant GemC1 in egg extracts (Figure 59A) it was important to determine whether this interaction had any effect on the activity of geminin. Human His₆-GemC1 and geminin proteins were co-expressed in bacteria and a stoichiometric complex was purified (This work was carried out by Christophe Caillat at The Netherlands Cancer Institute) (Figure 60C). Licensing inhibition assays were undertaken to determine the activity of various geminin-GemC1 complexes (Figure 60B). Extracts were released into interphase for 15 minutes to ensure the licensing reaction was active before being supplemented with a titration of geminin-GemC1 complexes. Extracts were incubated for 10 minutes before being supplemented with DNA and DNA replication was measured after 90 minutes. dGeminin dimers were very effective as licensing inhibitors, consistent with previous reports (Caillat et al., 2013), resulting in a complete inhibition of replication at all concentrations. Licensing inhibition assays were undertaken with dGeminin-dGemC1 complexes and this demonstrated that heterodimers inhibited licensing with an IC₅₀ of ~480 nM. Truncated heterodimers therefore have a significantly reduced ability to inhibit replication licensing compared to geminin homodimers. dGeminin-GemC1 complexes inhibited replication licensing at an IC₅₀ of 240-480 nM, suggesting these complexes were better inhibitors of licensing compared to dGeminin-dGemC1 complexes. However, dGeminin-GemC1 complexes were not present in stoichiometric amounts as some degradation of GemC1 was observed (Figure

60). Taken together, the data presented here suggests that GemC1 could potentially represent a novel geminin inhibitor, however it is unlikely to be present in *Xenopus* extracts at sufficient concentrations to effect geminin activity.

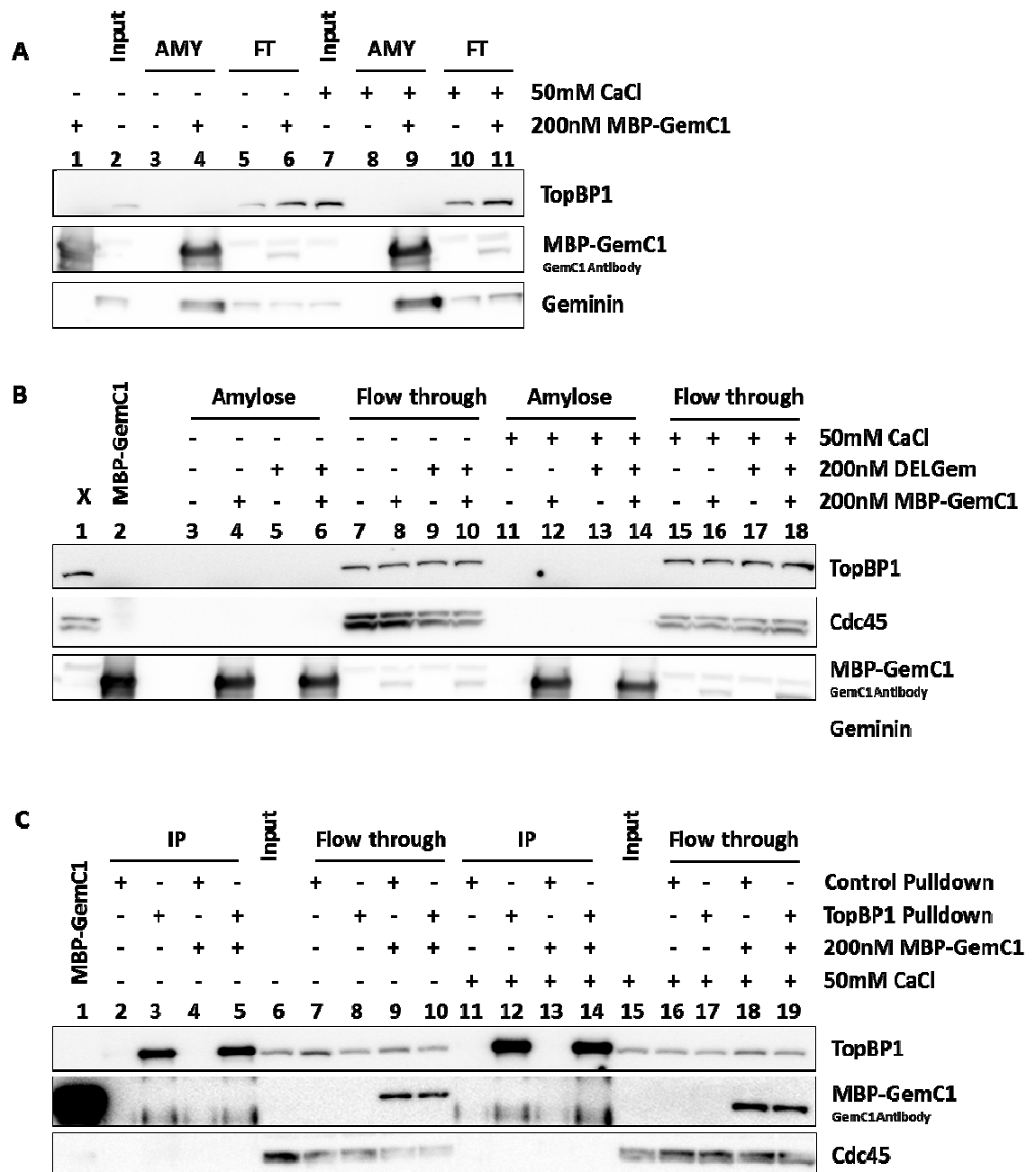


Figure 59: GemC1 Interactions by Pulldown and Immunoprecipitation. (A) Metaphase and interphase extracts were supplemented with 200 nM MBP-GemC1. MBP-GemC1 was pulled down with amylose resin (AMY) and samples were analysed by SDS-PAGE and western blot for TopBP1, MBP-GemC1 (Blotted with affinity purified-rabbit 1-97) and geminin. Input and flow through (FT) samples are shown to give an indication of protein depletion (B) Metaphase and interphase extracts were supplemented with 200 nM MBP-GemC1. MBP-GemC1 was pulled down with amylose resin (AMY) and samples were

analysed by SDS-PAGE and western blot for TopBP1, MBP-GemC1 (Blotted with affinity purified-rabbit 1-97) and Cdc45. Input and flow through samples are shown to give an indication of protein depletion. (C)TopBP1 was immunoprecipitated from metaphase and interphase extracts supplemented with 200 nM MBP-GemC1 using ProteinA DynaBeads crosslinked to affinity purified TopBP1 antibodies. Inputs, IPs and flowthrough was separated by SDS-PAGE and analysed by western blot analysis for TopBP1, Cdc45 and MBP-GemC1 (Blotted with affinity purified-rabbit 1-97).

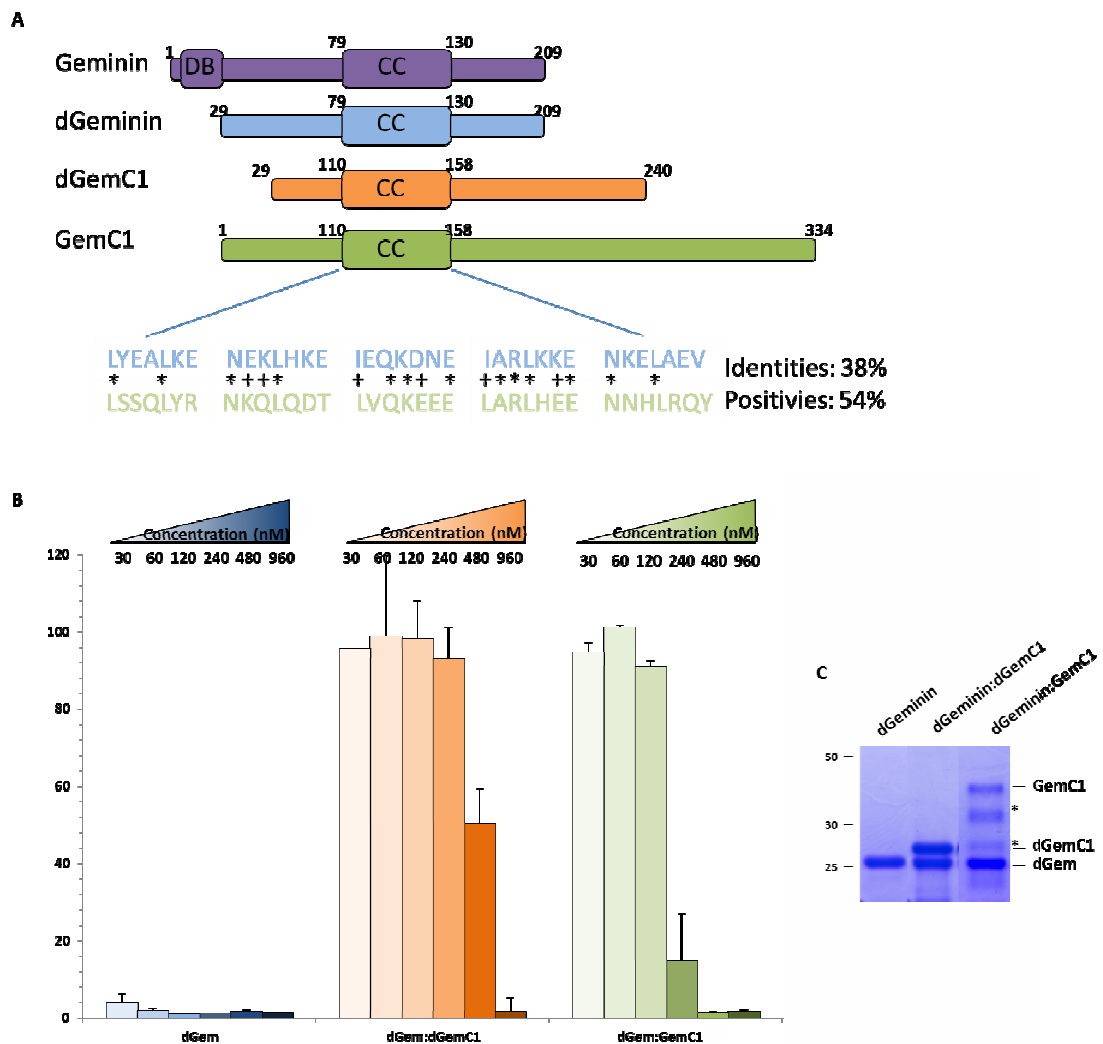


Figure 60: Assaying the activity of Geminin-GemC1 complexes. (A) Comparison of geminin and GemC1 proteins used in this study (DB= D-box, CC= coiled-coil). **(B)** Licensing inhibition assays: extracts were released into interphase for 15 minutes before being supplemented with various concentrations of geminin-GemC1 complexes. DNA was added after 10 minutes and replication was measured after 90 minutes. **(C)** Coomassie stain of proteins used in B. (degradation products are marked with an asterisk).

5.7 Summary

The main aim of this chapter was to investigate the potential role of protein-protein mediated interactions in the regulation of geminin activity. Experiments were undertaken to provide evidence for or against either of the hypothesised mechanisms: the sequestration of geminin by an inhibitor or the stoichiometric switch of geminin-Cdt1 complexes.

A switch like behaviour of the relative molar ratios of geminin and Cdt1 was observed which correlated well with the activation of licensing. However, this is unlikely to result in a switch from a permissive heterotrimer to an inhibitory heterohexamer; immunoprecipitation of geminin from interphase gel filtration fractions demonstrated that geminin and Cdt1 do not interact under these conditions and therefore it is unlikely that an active heterotrimer is formed in extracts. In fact, the molecular weight of the geminin-Cdt1 complex formed in metaphase extracts was estimated to be 143 kDa, suggesting that this complex is in fact a heterotrimer. Comparing the geminin-Cdt1 complexes that form in metaphase-arrested extract, PIE and NPE showed that the 143 kDa complex is the only geminin-Cdt1 complex that forms in extracts. This suggests that geminin can form a heterotrimer with Cdt1 to inhibit licensing or geminin is inactivated to break this complex and free Cdt1 can then take part in the licensing reaction.

All Cdt1-free geminin fractions identified by gel filtration were sensitive to high salt, suggesting that geminin exists in multiple forms or was present in different complexes. Active recombinant geminin elutes at the same volume as active metaphase geminin, and these were estimated by SEC-MALS and Siegel and Monty, respectively, to be geminin tetramers. Recombinant geminin never eluted at the same volume as either of the two interphase geminin complexes, even after the addition of salt. This suggests that

interphase geminin is biochemically different than active recombinant and metaphase geminin. The larger interphase complex was estimated to be slightly lower than a tetramer. This could be due to a change in the shape of the protein, or this complex may consist of novel geminin interacting proteins. Similar to this the estimated molecular weight of the smaller geminin interphase fractions did not fit well to a dimer or tetramer, suggesting that it had an altered shape or was part of a novel complex. This provides good evidence that geminin may be regulated by the interaction of novel proteins. It was demonstrated that GemC1 can bind strongly to geminin in extracts and that geminin-GemC1 complexes are ineffective licensing inhibitors compared to geminin alone, suggesting that GemC1 may be a novel geminin regulator; however, GemC1 is not likely to be present at high enough quantities in extract to carry out this role.

6.0 Discussion

6.1 Summary of Data Presented

Calcium addition facilitates a rapid destruction of geminin. Geminin levels fall to 50% within 15 minutes and the remaining protein is stable after this time with approximately 40% remaining at 90 minutes.

We have confirmed the previous reports that Cdt1 is subjected to DNA replication-coupled degradation and quantified this effect on endogenous protein using sperm DNA. DNA addition had no effect on the amount or kinetics of geminin degradation. Plotting molecular ratio of Cdt1 to geminin over time demonstrates a switch which correlates well with reports on the timing of licensing activation.

We have shown by co-immunoprecipitation and gel filtration studies that geminin and Cdt1 interactions change significantly at the metaphase to anaphase transition, consistent with published data. The gel filtration columns used had good resolving power resulting in the identification of four potentially unique geminin complexes. Co-immunoprecipitation and gel filtration studies were also used to demonstrate the interactions of endogenous geminin with recombinant Cdt1 and endogenous Cdt1 with recombinant geminin. These studies have shown that interphase geminin is unable to interact with Cdt1, therefore it is inactive.

We have confirmed previous reports that show that the 26S proteasome is not required for geminin inactivation.

Replication licensing is activated almost immediately after calcium addition and activity increases linearly until a maximum is reached at 10-12 minutes after calcium addition.

We have demonstrated that ongoing APC/C activity is required for licensing activity until a certain threshold is reached. Approximately 8 minutes after calcium addition ongoing APC/C activity is no longer required for licensing activity and a switch in APC/C dependence is observed. This was independent of CDK inactivation and does not require protein degradation, implying that 8 minutes of continuous APC/C activity is required to inactivate geminin, and after this timepoint APC/C E3 ubiquitin ligase activity is no longer required.

The depletion of free ubiquitin results in a failure to activate the licensing system. More specifically K11 linked ubiquitin chains are required for the activation of replication licensing. However, we have also provided evidence to suggest that DUBs are not required for the reactivation of geminin and therefore it is likely that direct ubiquitination is not a prerequisite for geminin inactivation during the metaphase-anaphase transition.

We have identified multiple geminin post-translation modifications by mass spectrometry and demonstrated that geminin can be SUMOylated *in vitro*.

Analysis of metaphase-arrested cytoplasm and S-phase nucleoplasm by gel filtration suggests that only one type of geminin-Cdt1 complex forms in extract and this complex has been estimated to be 143 kDa, which is likely to be a heterotrimer. In addition, co-immunoprecipitation of gel filtration fractions was used to demonstrate that geminin and Cdt1 do not interact in interphase extracts, suggesting that only free Cdt1 is active for licensing.

Active recombinant and endogenous geminin complexes were estimated by SEC-MALS and Siegel and Monty respectively to form tetramers, which were sensitive to high salt. Interphase geminin complexes were distinctly different than active geminin complexes

and could not be reconstituted with recombinant geminin. This suggests that interphase geminin has been altered in some way compared to active metaphase geminin.

The distinct difference between metaphase and interphase geminin complexes could be accounted for by the interaction of a geminin inhibiting protein. GemC1 is a geminin interacting protein that could potentially play a role in the regulation of geminin, however it is likely only present in extracts at a very low concentration.

6.2 Multiple Hypothesised Mechanisms

The aim of this project was to determine the molecular mechanism that accounts for the stabilisation and inactivation of geminin in *Xenopus* egg extracts. Geminin is a coiled-coil domain-containing protein that inhibits Cdt1 through protein-protein interactions to prevent DNA replication licensing. In somatic cell cycles the mechanism of geminin regulation is relatively simple; it is either active or completely degraded by the proteasome. In embryonic cell cycles only a portion of geminin is destroyed, therefore these cell types must regulate licensing by turning geminin off and on. Hypothetically, a mechanism for turning geminin activity on and off should be straight forward; a cell cycle controlled and reversible way of preventing geminin's interaction with Cdt1 is required. An on/off mechanism such as this is a common requirement for many cell cycle regulated proteins and mechanisms that are generally implemented to achieve this include direct protein post-translational modification, protein-protein interactions or regulation of subcellular localisation. The mechanism of geminin inactivation could hypothetically be straightforward, but in reality this is not the case. The enigmatic nature of this mechanism is highlighted by the number of wide ranging models that have been hypothesised to account for geminin inactivation.

The four basic models are shown (Figure 61). The following sections discuss how data obtained during this project fits into the multiple proposed models and a new model for the inactivation of geminin is shown based on the data during this project.

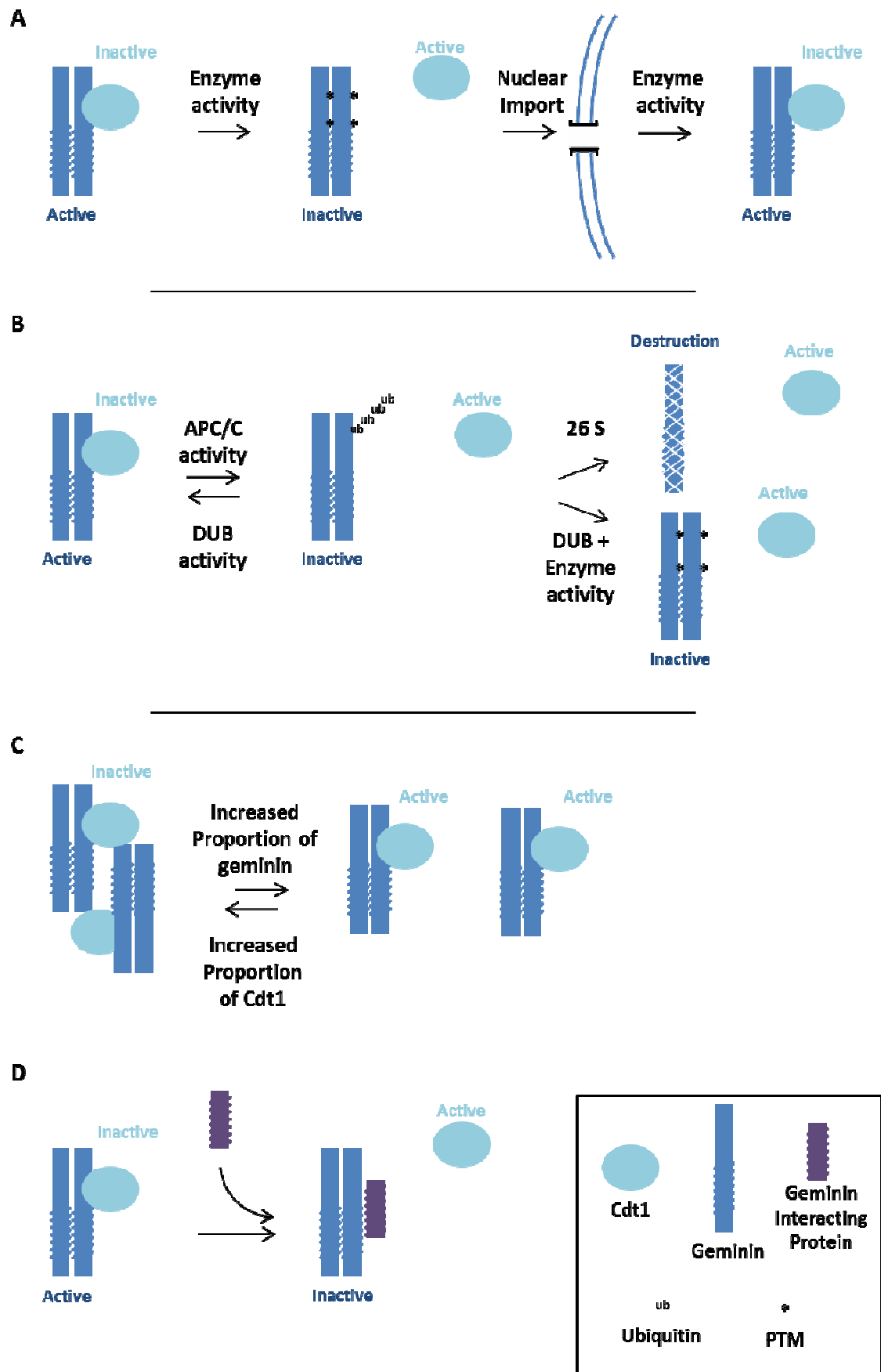


Figure 61: The Basic Models of Geminin inactivation. (A) Regulation by post-translational modification. Geminin is subjected to inactivating modifications to release free Cdt1 to take part in the

licensing reaction. Geminin is imported upon nuclear formation where it is reactivated in the nucleus. **(B)** A second PTM Model: the APC/C dependent inhibition of geminin. Activation of the APC/C by calcium addition leads to the ubiquitination of geminin. This allows free Cdt1 to take part in the licensing reaction. Geminin is then subjected to proteasome mediated destruction, reactivation by DUBs, or DUBed and inactivated by an additional mechanism. **(C)** Regulation of geminin by a stoichiometric switch in complex formation. Changes in the relative molecular ratios of geminin and Cdt1 could provide a dynamic switch to control protein activity. **(D)** Inhibition of geminin by a novel interacting protein. Activation of a cell cycle regulated geminin inhibitor protein could sequester geminin away from Cdt1 to facilitate licensing.

6.3 The Role of Post Translational Modifications

~50% of endogenous geminin remains stable in interphase extracts. The APC/C is shown to remain active as it continues to degrade Cdt1, therefore this stabilisation is geminin specific and is not simply due to the loss of E3 ubiquitin ligase activity over time. This predicts that there must be a mechanism to stabilise geminin in interphase extracts. A number of geminin post translational modifications were identified. The most interesting of these were a cluster of methylated and acetylated residues centred on the ubiquitination initiation motif of geminin- ⁵⁰RTK-KRK⁶². This motif has been shown to generate an intrinsic signal, separate to the D-box, that makes geminin a particularly efficient target of the APC/C (Williamson et al., 2011). Mutation of these charged residues to alanine leads to the stabilisation of geminin during *in vitro* APC/C ubiquitination reactions (Williamson et al., 2011) and also in *Xenopus* egg extract (Benjamin et al., 2004). Therefore it is likely that these post translational modifications are responsible for the stabilisation of geminin in interphase egg extracts. Approximately 50% of geminin is consistently destroyed. This matches well to mass spectrometry data, as 60% of metaphase peptides were acetylated at K62. This also correlates well with 2D gels electrophoresis data that shows multiple spots of various intensities. Therefore, a mechanism whereby geminin is stabilised in interphase egg extracts through modification of the ubiquitin initiation motif is proposed.

Showing that DUBs were not required for the inactivation of geminin suggests that geminin is regulated by mechanisms other than ubiquitination. Identification of multiple spots on 2D gel electrophoresis, identification of modified peptides by mass spectrometry and the observation that interphase geminin complexes may have an altered hydrodynamic radius all suggest that geminin activity may be regulated by PTMs. However, no modifications were observed on or around key sites on the protein. One explanation for this could be that the peptides were not identified due to the modification itself. In line with this a specific peptide between the primary and secondary interface was never identified which could have a functional significance. A region covering the tertiary Cdt1 interaction site was also specifically lost specifically in interphase samples.

If geminin is subjected to a number of post-translational modifications which are required for different outcomes i.e, stabilisation and/or inactivation, then it is likely there is a mechanism to target a specific portion of the protein for neither or both modifications. It could be proposed that Cdt1 would either target geminin for destruction to enable a rapid activation of licensing, or for stabilisation/inactivation to maintain relative amounts of geminin for S-phase and the following mitosis. This is analogous to the dual function of geminin that is carried out in G2 and mitosis in human cells to stabilise Cdt1 (Ballabeni et al., 2013). It has been shown that Cdt1 depleted metaphase extracts that are stimulated to with calcium do not destroy significant levels of geminin, suggesting that Cdt1 targets geminin for destruction (Maiorano et al., 2004). This was tested by supplementing metaphase extracts with recombinant Cdt1 prior to release into interphase, however these preliminary results were inconclusive.

6.4 The Role Protein-Protein Interactions

Experiments undertaken to determine whether geminin could be regulated by a stoichiometric switch demonstrated that no interaction can be detected between geminin and Cdt1 in interphase. This suggests that only free Cdt1 can participate in the licensing reaction. The idea that a geminin-Cdt1 complex could be active for licensing first came about when a co-expressed geminin^{DEL}-Cdt1 complex added to extract was shown to be equally active compared to Cdt1 alone (Lutzmann et al., 2006). This disagreed with earlier work carried out in *Xenopus* egg extracts that resulted in the reconstitution of replication licensing. Licensing factor activity could be reconstituted with purified Cdt1 or recombinant Cdt1 and this was blocked by the addition of geminin (Gillespie et al., 2001). In addition, fractionation of partially purified endogenous Cdt1 on phenyl sepharose was able to separate free interphase Cdt1 from geminin-bound interphase Cdt1. These fractions were assayed for their ability to rescue replication in Cdt1 depleted extracts and it was conclusively shown that only free Cdt1 could efficiently rescue replication (Tada et al., 2001). The Geminin-Cdt1 complex size was consistent across various extract types, and this complex was likely a heterotrimer. However, this work did not rule out the possibility that higher molecular weight complexes can form, as all gel filtration was carried out in relatively high salt buffer (200 mM NaCl). Observing the molar ratios of geminin and Cdt1 in extract throughout the replication cycle of *Xenopus* egg extract provided compelling evidence for the existence of a protein concentration dependent switch in licensing activity. Therefore a mechanism could be hypothesised where geminin and Cdt1 do not interact at all at a high ratio of Cdt1 to geminin, but form complexes at high concentrations of geminin. While this mechanism remains possible, it is based on an observation rather than experimental evidence, and the switch in molar ratio may be a cause or consequence of replication licensing.

The estimation of geminin and Cdt1 complexes sizes by Siegel and Monty and experiments demonstrating the instability of geminin complexes in high salt suggest that interphase geminin may be inactivated by an interacting protein. Licensing assays carried out using geminin-GemC1 complexes showed that GemC1 has a significant effect on the ability of geminin to inhibit licensing and therefore has good potential to be a geminin regulating protein

6.5 The Role of the APC/C During Replication Licensing Activation

The only essential function of the APC/C for the activation of replication licensing in *S. cerevisiae* is the inactivation of CDKs (Noton and Diffley, 2000). We have shown in a Metazoan model that ongoing APC/C activity is essential for 8 minutes to activate licensing and after this timepoint APC/C E3 ubiquitin ligase activity is no longer required. This shows that higher eukaryotes contain an APC/C regulated protein that inhibits DNA replication licensing that is not present in yeast cells. This protein is geminin (Li and Blow, 2004). Therefore, the inactivation of geminin specifically requires at least 8 minutes of APC/C E3 ubiquitin ligase activity.

Three essential requirements have been identified during this project for the activation of replication licensing: 8 minutes of APC/C E3 activity; free ubiquitin and the ability to form K11 linked ubiquitin chains on substrate proteins. These requirements can all be deduced to be specifically required for geminin inactivation. 8 minutes of APC/C E3 ligase activity is required in the presence of CDK inhibitors, therefore this activity is required to inhibit geminin; K11 linked ubiquitin chains are essential for licensing activation, therefore ubiquitin is essential and this is likely to be specific for geminin inactivation as it has been shown that cyclin-B inactivation can occur in the absence of chain by multiple mono-ubiquitination (Dimova et al., 2012). Taken together, 8 minutes of APC/C K11 linked ubiquitin chain formation is required either directly or indirectly

to inactivate geminin. Once this has occurred the APC/C is no longer required. In addition two functions that are dispensable for replication have been identified during this project, proteasomal degradation and DUB activity. While specific ubiquitin chain types are essential the 26S proteasome is not essential for licensing activation. It is important to highlight the implications of the non-essential nature of DUBs, DUBs were found to be dispensable for the reactivation of geminin after nuclear import. This implies that geminin is not inactivated by direct ubiquitination.

Taking this together I propose that geminin is inactivated by two separate APC/C dependent mechanisms, first by direct ubiquitination to rapidly inactivate geminin in response to calcium, and second by an indirect mechanism as the APC/C is no longer required after 8 minutes. It is likely that there is an APC/C substrate that is regulated during the metaphase-anaphase transition albeit with slower kinetics than CDK and geminin, which then acts to inhibit geminin. This explains why licensing can still be activated without the proteasome and without APC/C after a certain time; the APC/C is required to temporarily inhibit geminin while also activating a geminin inhibitor, either directly or indirectly. This mechanism takes all observations into account. Ongoing APC/C activity is required until the second mechanism is activated because Cdt1 activation in the absence of ongoing APC/C activity is labile, there is an additional pool of free geminin capable of binding to and inhibiting Cdt1. Assuming the K11 linked chains are sufficient to break the interaction of geminin and Cdt1, ongoing APC/C activity is sufficient even in the absence of protein destruction as the second mechanism is unaffected by loss of the proteasome and mediates geminin inactivation, resulting in an irreversible change in the requirement of the APC/C and the activation of licensing.

This model is supported by data that shows bulk geminin ubiquitination occurs within 10 minutes of calcium addition after which point high molecular weight species of

geminin are not observed (Li and Blow, 2004). Data presented in this thesis has shown that interphase geminin has distinct properties compared to activate metaphase geminin which could represent a protein interactor or geminin modification consistent with the second step in the APC/C-dependent mechanism.

A good candidate for this hypothesised modifier/interactor would be a CDK regulated protein. Inhibition of the modifier/interactor by high CDK in mitosis would ensure geminin activity. CDK activity leads to APC/C activation which in turn rapidly destroys mitotic cyclins to inhibit CDK. Phosphatases become prevalent and remove mitotic phosphorylations as extracts enter interphase. Dephosphorylation of the modifier/interactor after 8 minutes would lead to the inactivation of geminin. Nuclear important later in the cell cycle would reactivate geminin as the geminin modifier is once again inhibited by high CDK activity in the nucleus.

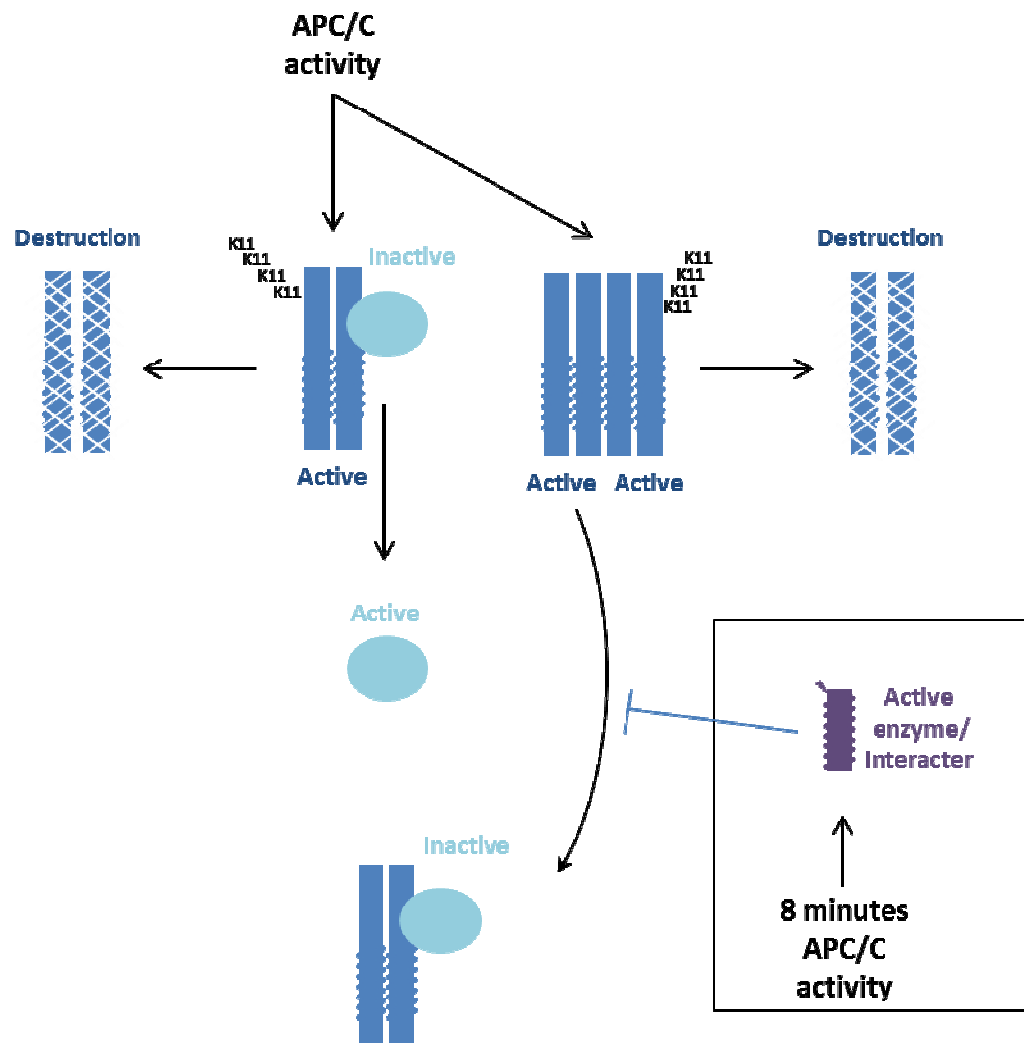


Figure 62: Geminin is regulated by the APC/C via Two Distinct Mechanisms This is a model based on all the observed data of this thesis. The activation of the APC/C results in an immediate conjugation of K11 linked chains on all types of geminin complexes. Assuming that K11 chains can inhibit geminin activity before destruction, then APC/C activity starts to accumulate free Cdt1, resulting in the linear activation of licensing. Extracts, however, have a large pool of active geminin. Therefore this extra pool of geminin can bind to and inhibit the free Cdt1. This makes the activation of Cdt1 labile and makes the initial 8 minutes of APC/C activity reversible. The interaction of this free geminin with activate Cdt1 is likely to be responsible for the loss of licensing activity at early timepoints during D-box assays. While geminin and CDK are the two main targets known for the regulation of replication licensing, they are not necessarily the main targets for APC/C ubiquitin activity. Therefore while the APC/C mediates the initial activation of licensing by modifying geminin it also mediates activation of a second geminin inhibiting pathway. After 8 minutes this second geminin inactivator is reversibly active to inhibit geminin and therefore the APC/C is no longer required to maintain licensing activity.

7.0 Conclusion and Future Outlook

The work presented in this thesis has focused on a characterisation of the mechanisms that inactivate and stabilise geminin in *Xenopus* egg extracts. Whereas some progress has been made in determining the key players that facilitate these outcomes, further work is required to demonstrate how each step in the molecular mechanism is undertaken. In the future it would be of interest to develop an assay that would allow recombinant geminin to become inactivated by extracts. This would allow for a mutational analysis of sites to determine what residues are required for geminin inactivation and reactivation by observing the ability to form specific complexes in extract or PIEs. In addition would be interesting to further investigate the mechanism of geminin G1 stabilisation in mouse and human embryonic cells by mutating the ubiquitination initiation motif and searching for post translational modifications by mass spectrometry. In light of my proposed speculative mechanism for the role of the APC/C it would be interesting to do a ‘big data’ experiment to characterise all the APC/C targets at the metaphase-anaphase transition. This could be carried out using UbVS to deplete ubiquitin, rescuing the extract with His6-Ubiquitin and performing denaturing Ni-NTA pulldowns at various times after calcium addition. Samples could then be prepared by sequential FASP to identify multiple post translational modifications such as ubiquitination, acetylation and phosphorylation.

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